

IRON HOMEOSTASIS AND ANEMIA IN HIV AND TUBERCULOSIS

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Background: Evidence supports a role for iron homeostasis in the pathogenesis of HIV and tuberculosis. The iron regulatory peptide, hepcidin, inhibits both dietary iron absorption and iron efflux leading to macrophage iron retention. Conditions associated with elevated hepcidin, which include altered concentrations of ferritin, transferrin and hemoglobin, may favor *Mycobacterium tuberculosis* iron acquisition, HIV progression and anemia. In light of this, we investigated iron homeostasis biomarkers as risk factors for incident TB and mortality in an HIV-seropositive cohort and also for progression to TB in an HIV-seromixed cohort. In addition, we characterized iron homeostasis during TB therapy in order to identify causes of anemia and inform selection of safe, effective and well-timed treatments for anemia.

Methods: Clinical and demographic data and archived plasma and serum samples from two research platforms were utilized in this work: the Medical Research Council (MRC) HIV clinical cohort and the MRC TB case contact study. Analysis of iron homeostasis biomarker concentrations was conducted at MRC laboratories in The Gambia.

Results: During HIV Clinical Cohort follow up, 32 incident tuberculosis cases were identified and 64% of the 196 participants died. Greater hepcidin was associated with significantly increased likelihood of tuberculosis and greater all-cause mortality. This was consistent with observed higher ferritin and hepcidin concentrations in HIV-seromixed contacts of infectious TB cases that progressed to TB earlier. Analysis of biomarkers and anemia during TB therapy revealed that anemia of inflammation was predominant TB diagnosis, declining significantly after six

months of treatment; however, a corresponding reduction was not evident for anemia with iron-responsive components. Hepcidin concentrations significantly declined after 2 months of TB treatment.

Conclusions: Changes in iron homeostasis biomarkers are associated with incident TB and mortality in HIV and progression to TB disease among contacts of infectious TB cases. Further studies are needed to elucidate mechanisms and determine the clinical utility of monitoring iron homeostasis biomarkers. TB therapy is associated with significant reductions in anemia of inflammation, but iron-based interventions are needed for anemia with iron responsive components. Monitoring hepcidin reveals a window for intervention opening as early as two months into TB treatment.

BIOGRAPHICAL SKETCH

Peter Minchella was born and raised just down the road from Purdue University in West Lafayette, Indiana. Upon graduating from high school, he enrolled at Colgate University in Hamilton, New York to study molecular biology and play basketball at the National Collegiate Athletic Association (NCAA) Division 1 level. Peter earned four varsity letters and his B.A from Colgate in 2008 and shortly thereafter boarded a flight for Mozambique, where he had been assigned to serve as a Peace Corps Volunteer. During his 27 month Peace Corps service, Peter taught chemistry and biochemistry courses, coordinated a science fair project, and worked on HIV prevention and awareness initiatives. Peter's time in Mozambique exposed him to healthcare, nutrition and agricultural challenges not faced in the United States and motivated him to pursue further education that would allow him to better understand these challenges and enable him devise strategies to overcome them.

Peter matriculated into the PhD program in international nutrition at Cornell University in 2011. During his doctoral studies, he conducted his dissertation research in The Gambia, where he worked in collaboration with the Medical Research Council (MRC) International Nutrition Group and the MRC Tuberculosis (TB) Immunology Group.

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TABLE OF CONTENTS

| | |
|---|-----|
| List of Figures | v |
| List of Tables | vii |
| CHAPTER 1: Introduction..... | 1 |
| Iron (regulation, assessment, anemia, acute phase response) | |
| Iron and Infection | |
| HIV (prevalence, pathogenesis, treatment, role of iron) | |
| TB (prevalence, pathogenesis, treatment, role of iron) | |
| HIV/TB co-infection (prevalence, pathogenesis, treatment, role of iron) | |
| Research Objectives | |
| Setting and study population | |
| References | |
| CHAPTER 2: Elevated hepcidin at HIV diagnosis is associated with incident tuberculosis in a retrospective cohort study..... | 24 |
| Abstract | |
| Introduction | |
| Methods | |
| Results | |
| Conclusions | |
| CHAPTER 3: Links between hepcidin, iron homeostasis, inflammation and mortality in HIV infection | 33 |
| Abstract | |
| Introduction | |
| Methods | |
| Results | |
| Conclusions | |
| CHAPTER 4: Iron homeostasis and progression to pulmonary tuberculosis disease among household contacts | 70 |
| Abstract | |
| Introduction | |
| Methods | |
| Results | |
| Conclusions | |

| | |
|--|-----|
| CHAPTER 5: Complex anemia in tuberculosis: the need to consider causes and timing while designing interventions | 93 |
| Abstract | |
| Introduction | |
| Methods | |
| Results | |
| CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS | 116 |
| Conclusions | |
| Recommendations for Future Research | |
| APPENDIX | 123 |
| Roles of Iron | |
| Conference Abstracts | |
| Keystone Symposia (2013) | |
| International AIDS Society (2013) | |
| Experimental Biology (2014) | |
| Standard operating procedures (SOPs) | |
| Hepcidin | |
| Ferritin | |
| Transferrin | |
| Soluble Transferrin Receptor | |

LIST OF FIGURES

| | |
|---|-----|
| Figure 2.1. Incident tuberculosis classified by hepcidin quartiles at HIV diagnosis..... | 28 |
| Figure 2.2. Plasma hepcidin at HIV diagnosis and additional baseline risk factors in relation to incident tuberculosis..... | 29 |
| Figure 3.1. Scatterplot between circulating hepcidin concentration at HIV diagnosis and relevant variables | 51 |
| Figure 3.2. Box-plots of circulating hepcidin concentrations | 54 |
| Figure 3.3. Kaplan-Meier survival curves according to hepcidin tertiles..... | 55 |
| Figure 3.4. Pathways to HIV-associated anemia..... | 62 |
| Figure 4.1. Study Flowchart..... | 75 |
| Figure 4.2. Iron homeostasis at enrollment among active tuberculosis cases prior to treatment initiation and household contacts that became early TB-progressors, delayed TB-progressors or remained non-progressors..... | 82 |
| Figure 5.1. Risk factors for all-cause anemia and cause-specific anemia..... | 106 |
| Figure 5.2. Iron status biomarkers in active TB cases at TB diagnosis, <i>Mycobacterium tuberculosis</i> Complex infected (TST-positive) and uninfected (TST-negative) contacts..... | 107 |
| Figure 5.3. Longitudinal changes in ferritin, hepcidin, soluble transferrin receptor (sTfR), transferrin and hemoglobin concentrations among TB cases during treatment..... | 108 |

LIST OF TABLES

| | |
|--|-----|
| Table 3.1. Anemia in HIV infection in Africa..... | 38 |
| Table 3.2. Characteristics of cohort participants at HIV diagnosis..... | 49 |
| Table 3.3. Cox regression models of hepcidin, iron homeostasis and inflammation and associations with all-cause mortality in HIV infection..... | 56 |
| Table 3.4. Principal components analyses of iron homeostasis and inflammatory variables and Cox regression analyses of all-cause mortality..... | 58 |
| Table 4.1. Enrollment characteristics of age-eligible TB-progressors and non-progressors..... | 79 |
| Table 4.2. Poisson regression models of the association between standardized iron homeostasis biomarkers, standardized body mass index ^a and progression to pulmonary tuberculosis..... | 85 |
| Table 5.1. Participant characteristics amongst at study recruitment and amongst TB cases at TB diagnosis and following two and six months of TB treatment..... | 103 |
| Table 5.2. Predictors of anemia etiologies..... | 105 |

CHAPTER 1

Introduction

Iron

Iron (chemical symbol: Fe) is one of the most abundant elements in the earth's crust and an essential nutrient for almost all known organisms. It is able to receive and release electrons during conversion from Fe(II) to Fe(III) and plays a major role in DNA production and in energy generation (1). In humans, iron is considered indispensable because it serves as a co-factor for many proteins, including hemoproteins and enzymes, which are essential for fundamental cellular processes (2).

At a given time, between three and five grams of iron are present in the human body, with the majority in the form of heme in hemoglobin. Macrophages, muscle myoglobin, and the liver parenchyma also contain significant portions of body iron. Absorption of dietary inorganic iron occurs through enterocytes via divalent metal transporter 1 (DMT1) following reduction of Fe(III) to Fe(II). Internalization of dietary heme also occurs through enterocytes, though the mechanism is not well defined. Export of iron from enterocytes is mediated by ferroportin and coupled with re-oxidation from Fe(II) to Fe(III) by either the ferroxidase hephaestin or its homologue, ceruloplasmin. Once exported, iron binds to the iron transport protein, transferrin, for delivery to tissues and erythroblasts. Macrophages acquire iron via erythrophagocytosis.

Regulation and assessment of iron status/iron homeostasis

The potential consequences of "too much" or "too little" iron in the human body are dire. They include oxidative damage, anemia and increased susceptibility to infection. To avoid

these consequences, a system of highly regulated mechanisms is in place to control iron homeostasis. Regulation occurs at both the systemic and cellular levels and influences a number of iron-associated proteins including the five proteins that are the focus of this work: ferritin, hepcidin, transferrin, soluble transferrin receptor (sTfR) and hemoglobin. The iron-related functions of these proteins and their biological mechanisms of effect related to iron homeostasis are summarized in Appendix 1.

There are a plethora of methods available to assess iron status and/or iron homeostasis in clinical and research settings. Measurement of serum/plasma iron, zinc protoporphyrin (ZnPP), and transferrin saturation are part of a long list that also includes assessment of concentrations of ferritin, hepcidin, transferrin, soluble transferrin receptor (sTfR) and hemoglobin. Of the five proteins that are the focus of this work, the iron storage protein ferritin and oxygen transport protein hemoglobin are the most commonly measured. However, sTfR has important clinical utility when inflammation is present, and both transferrin and hepcidin are emerging as informative markers.

Anemia

When hemoglobin concentration falls below a certain cutoff (which varies by age, sex and race (3)), oxygen delivery to tissues is considered to be impaired and the individual with low hemoglobin is considered to be anemic. Anemia affects an estimated one-quarter of the world's population (4), with a disproportionate share of cases occurring in sub-Saharan Africa (5). Consequences of anemia include increased morbidity and mortality (6), decreased quality of life (6), and diminished productivity (7). Causes of anemia are diverse and often act in combination with each other. While the largest worldwide contributor to anemia is thought to

be iron-deficiency (4), factors including hemoglobinopathies, other micronutrient deficiencies and infection also play important roles in contributing to the worldwide anemia burden.

While determinants of iron deficiency anemia (IDA) are diverse (e.g., parasitic worms, diet, pregnancy) (8), the mechanisms responsible for IDA are straightforward: either decreased iron supply impairs hemoglobin production, or bleeding causes the loss of erythrocytes faster than they can be replaced. Other factors that contribute to and cause anemia do so via more complex pathways. Anemia of inflammation (AI) (also referred to in the literature as “anemia of chronic disease (ACD)”), is considered to be the second largest contributor to anemia after iron deficiency (9). It is a particularly important factor to consider in settings where anemia co-exists with high rates of infection. AI arises as a result of an innate immune response, in which pattern-recognition receptors on monocytes, macrophages, neutrophils and dendritic cells recognize motifs specific to pathogens and release inflammatory cytokines. The resulting inflammation leads to general alteration of iron homeostasis as well as anemia-causing pathologies including short erythrocyte life-span, poor erythrocyte iron incorporation and decreased sensitivity to or supply of erythropoietin (10).

The iron regulatory protein, hepcidin, plays an important role in the mechanisms responsible for AI and the inflammation-mediated alteration of iron homeostasis. Specifically, hepcidin binds to and degrades the iron export protein ferroportin and down-regulates expression of the iron importer DMT1. Since hepcidin transcription is induced by the pro-inflammatory cytokine, interleukin-6 (IL-6), inflammation leads to a reduction in iron absorption and causes iron to be sequestered in macrophages and enterocytes. This mechanism, which is supported by numerous *in vitro* studies (11), contributes to the overall alteration of iron

homeostasis that is a hallmark of AI and also limits iron delivery to erythroblasts, functionally causing anemia. Studies conducted in humans support this mechanism and also indicate that hepcidin is a predictor of both erythrocyte iron incorporation (12) and the effectiveness of oral iron therapy (13).

Acute Phase Response

In addition to lower hemoglobin concentrations, the inflammatory cytokines that are released upon innate recognition of a pathogen induce changes in plasma concentrations of other proteins. This phenomenon is referred to as the acute phase response (APR) and the affected proteins are considered acute phase proteins (APPs). The iron homeostasis proteins ferritin, transferrin and hepcidin are considered APPs, as are C-reactive protein (CRP) and alpha-1-antichymotrypsin (ACT), both of which are often utilized in studies as markers of inflammation. Changes in APPs must be considered when interpreting concentrations of iron homeostasis proteins. For example, a study in rural Zambian children reported that measurements of serum ferritin were 279-356% higher for those experiencing infection (14).

Assessing iron homeostasis while also controlling for changes in APPs is a challenge. However, innovative indices have been developed that enable researchers and clinicians to interpret APPs even when inflammation is present. For research or clinical work involving iron homeostasis proteins the non-APP, soluble transferrin receptor (sTfR), is often utilized. Soluble transferrin receptor (sTfR) is a cleaved portion of transferrin receptor, which is a carrier protein necessary for cellular internalization of transferrin. Since sTfR is regulated primarily by intracellular iron concentrations, it can be incorporated into indices in conjunction with iron homeostasis proteins that are APPs to assess iron status. These indices are especially handy in

situations where it is important to determine if an apparently iron deficient or sufficient individual is actually iron deficient/sufficient or if inflammation is an influencing factor. Specifically, the sTfR/log(ferritin) ratio has been widely used as a means by which to distinguish AI from IDA and other types anemia (15).

Iron and Infection

In the context of infection, iron's limited availability within the human body and its physiological importance to both hosts and microbes make it a valuable commodity. Many microbes depend on host-acquired iron and, in response; hosts use their complex system of iron regulation to modify their iron metabolism and restrict iron availability. Microbes, in turn, employ complex iron acquisition strategies to obtain the restricted resource.

Since microbes cultured in iron-scarce environments show decreased energy and nucleic acid production (16) the prevailing "nutritional immunity" hypothesis (17) suggests that iron-deficient host environments are better equipped to resist infection than iron-rich host environments. While some evidence supports this hypothesis (18-20), the relationship is likely more complicated and dependent on the pathogen that is causing the infection. In general, it may be that a proverbial 'goldilocks zone' governs the relationship between iron status and infection. Iron deficiency or 'too little iron' is known to compromise cell-mediated immune function (21) and iron sufficiency or 'too much iron' increases iron availability to pathogens leading to increased disease susceptibility (18, 22). The optimal or 'just right' level of iron status, which has not been defined, may maintain immune function while also conferring some protection against pathogens.

Since this work focuses on iron homeostasis in human immunodeficiency virus (HIV) infection, HIV/tuberculosis (TB) co-infection and TB; the following sections will concentrate on the prevalence, pathogenesis, diagnosis and treatment of these diseases, as well as the research related to iron homeostasis in each context.

Human Immunodeficiency Virus (HIV) Prevalence

HIV is the world's leading cause of infectious disease mortality. It was first identified in 1980 and it has claimed approximately 36 million lives since. There are currently 35 million people living with HIV, the majority of whom reside in sub-Saharan Africa (23). Worldwide, HIV prevalence ranges from approximately 26% of adults aged 15-49 in Swaziland to <0.1% in Afghanistan (23).

Human Immunodeficiency Virus (HIV) Pathogenesis

HIV is transmitted from human to human via bodily fluids. Upon entrance into the body, the virus identifies and binds to a target cell. Following binding, HIV's viral envelope fuses with the target cell membrane allowing the HIV capsid and genome to enter the cell. Once inside, HIV utilizes reverse transcription to convert its genetic material from RNA to DNA, which allows it to enter the nucleus where it is integrated into the host cell's DNA. At this point, HIV is able to use host cell machinery to create HIV proteins, which are assembled into new HIV viruses and released. Due to the presence of viral DNA, many cells that are/were targeted by HIV for replication sacrifice themselves in an inflammatory process known as pyroptosis (24). Since HIV preferentially targets human CD4+ T cells for replication, many of the cells destroyed are CD4+ T cells. This is important because the CD4 receptors on these cells typically work in combination

with T cell receptors on antigen presenting cells to coordinate immune system responses to antigens. When CD4+ T cells are destroyed, the ability of the immune system to respond to antigens is greatly impaired.

During initial acute HIV infection, HIV is able to replicate quickly and many CD4+ T cells are destroyed. This phase is short-lived, however, and is typically countered by an immune response that leads to an increase in CD4+ T cell numbers and transition into a period of clinical latency. During latency, HIV reproduces at very low levels, which slowly decreases the host's CD4+ T cell count, but does not result in clinical symptoms. Latency lasts an average of ten years without treatment and can last much longer with treatment (25). When the CD4+ T cell count is depleted to less than 200 CD4+ T cells/microliter, the HIV infection is considered to have progressed to Acquired Immunodeficiency Syndrome (AIDS). At this point the ability of the immune system to respond to opportunistic infections is severely impaired. Without treatment, people who progress to AIDS are unlikely to survive for more than 3 years (25).

Drivers of HIV infection are complex and multifactorial. While CD4+ T cell depletion is typically utilized to track HIV progression, interconnected factors including viral replication, immune activation, inflammation and microbial translocation are all involved in driving progression of the infection. Anti-Retroviral Therapy (ART), which is a combination therapy that targets different stages of the HIV life cycle, is currently the best option for slowing HIV progression. However, strategies such as prophylaxis against opportunistic infections and micronutrient supplementation (26) have also been shown to extend lives and reduce the risk of immune-decline in HIV-infected people.

Role for Iron in HIV

Iron is required for several steps in the life cycle of HIV, including reverse transcription, gene expression and capsid assembly (27). Emerging evidence from both human and *in vitro* studies suggests that iron is an important regulator of HIV progression and that elevated iron status leads to more rapid progression of infection. Specifically, both hereditary iron overload (28) and high iron status (29) appear to speed up progression of HIV, while iron chelation slows it down (30). Similarly, *in vitro* expression of the iron exporter, ferroportin, was associated with reduced levels of HIV transcription (31) and increased levels of iron in primary CD4+ T cells have been associated with increased HIV replication (20).

More broadly, in an ART-naïve HIV-seropositive West African cohort a pattern of iron redistribution characterized by elevated ferritin, decreased transferrin and decreased hemoglobin (32, 33) was associated with mortality in HIV infection. This finding is consistent with a study linking high ferritin to mortality in another HAART-naïve HIV-seropositive cohort and also with studies indicating that anemia is a strong morbidity and mortality risk factor in HIV infection (34-38). At this point, the mechanisms that link anemia, iron redistribution and mortality in HIV infection are not well defined.

An additional factor that must be considered, but one that increases the complexity of the aforementioned mechanisms, is ART. The studies summarized in Table 3.1 indicate that ART reduces the prevalence of anemia in HIV-infected populations, but also suggest that anemia remains unresolved in many people even after initiating ART.

TB Prevalence

Estimates suggest that approximately one-third of the world's population is infected with TB causing bacteria, also known as the *Mycobacterium tuberculosis* (*Mtb*) Complex (MTBC).

While the vast majority of infected individuals will never progress from latent TB infection (LTBI) to active TB disease, TB is widespread and considered the world's second leading cause of infectious disease mortality. World Health Organization estimates for 2013 indicated that there were 9.0 million new cases of TB and 1.5 million deaths from TB worldwide, with the bulk of both cases and deaths occurring in Africa and Southeast Asia (39).

TB Pathogenesis

While there are several mycobacterial species that make up the MTBC and cause TB, most cases of active TB disease are attributed to *Mtb*. Transmission of *Mtb* occurs when an individual with active pulmonary TB disease coughs, sneezes, or shouts – liberating tiny airborne particles of tubercle bacilli that can be inhaled by anyone in close proximity. Inhaled bacilli make their way to the alveoli of the lungs where they are phagocytosed (internalized) by alveolar macrophages. Phagocytosis is followed by maturation of the phagosome, a process in which the chamber containing the *Mtb* bacilli is subject to acidification, production of reactive oxygen/nitrogen species, and release of anti-microbial peptides. Phagosomal maturation is often sufficient to destroy pathogens, but *Mtb*'s unique cell wall enables it to tolerate and in some cases impair the phagosomal maturation process (40, 41).

Infected macrophages move from the airways into pulmonary tissue where a local inflammatory response initiates formation of a granuloma. The granuloma is a hallmark of TB and is characterized by a core of infected macrophages surrounded by monocytes and T lymphocytes. In most people infected with *Mtb*, granuloma formation signals that the host immune system has mostly managed to halt the replication and spread of *Mtb*. This stage of pathogenesis is known clinically as latent TB infection (LTBI). During LTBI, the host immune

system keeps *Mtb* in check through deployment of phagosomal defense mechanisms and promotion of unfavorable conditions within the granuloma, while *Mtb* enters a state of dormancy in which its metabolic activity decreases and its ability to resist host defense mechanisms increases (42). LTBI is asymptomatic and may be short lived or last for the individual's remaining years of life.

While some individuals progress directly from *Mtb* infection to active TB disease, most experience some form of LTBI between infection and active TB disease (43). In the 5-10% (43) of individuals who progress from LTBI to active TB disease, dormant *Mtb* reactivates and begins replicating within the granuloma. Due in part to increased metabolic activity by *Mtb*, the granuloma's previously solid center becomes caseous and begins to lose its structure. Eventually the unstructured granuloma ruptures, releasing TB bacilli into the airways (42). The mechanisms responsible for reactivation of once-dormant tubercle bacilli are not well understood. Some studies have speculated that resuscitation-promoting factor (Rpf) plays a role in inducing reactivation (44). The *Mtb* genome contains several Rpf orthologs (44) and a 2010 study showed that addition of Rpf to sputum from TB patients increased recovery of *Mtb* (45). Another model suggests that 'scout' *Mtb* organisms may determine whether or not broader *Mtb* reactivation will occur by sensing the attractiveness of the environment for replication (42). In this model limitation of nutrients, including iron, would likely play an important role in determining the likelihood of *Mtb* reactivation.

TB Diagnosis and Treatment

Sustained high levels of new TB cases and deaths from TB serve as evidence that diagnosis and treatment of TB present major challenges. TB diagnosis has traditionally been

based on a “passive case finding” strategy, in which symptomatic individuals who presented at medical facilities were tested for the disease using sputum-smear microscopy and mycobacterial culture. While passive case finding, microscopy and culture are still in wide use, recent advances have improved case identification and accelerated TB diagnosis. In particular, community based case finding strategies (e.g., enhanced case finding, in which communities are sensitized about TB signs/symptoms and TB contacts are screened) and the Xpert MTB/RIF test have had recent impacts (46, 47). These developments are proving to be especially important in resource restricted settings where TB prevalence is high and access to labs outfitted for TB culture is decreased.

Upon diagnosis, current treatment guidelines for active TB disease include an intensive phase of the anti-TB drugs isoniazid, rifampicin, ethambutol, and pyrazinamide for two months, followed by isoniazid and rifampicin for four months (48). Isoniazid and ethambutol work against TB causing bacteria by interfering with mycobacterial cell wall production, rifampicin inhibits bacterial RNA synthesis and pyrazinamide impairs mycobacterial fatty acid synthesis. With timely diagnosis these drugs are extremely effective. Cure rates for patients (non-multi-drug resistant TB) on TB treatment are close to 90% (49). However, cure rates for untreated TB cases or for patients in whom treatment is delayed, are poor. 70% of adults with untreated PTB die within 10 years (50) and delayed PTB treatment is linked to increased risk of mortality (51). In addition, foregoing or delaying treatment fundamentally increases the likelihood of transmission of TB causing bacteria (52). Similar to patients who experience delays in TB treatment, TB patients with co-morbidities who are undergoing treatment are at higher-risk for poor TB outcomes. Anemia at TB diagnosis, for example, has been linked to an increased risk of

death (53, 54). The same is true for individuals with malnutrition (55), bacterial pneumonia (54), and HIV/TB co-infection (56). Thus, timely diagnosis, swift administration of treatment, and resolution of co-morbidities are key components in reducing TB transmission and death from TB.

LTBI Screening and Treatment

The standard screening method for LTBI is the mantoux tuberculin skin test (TST). The TST is administered as an intradermal injection of a standard volume of tuberculin purified protein derivative (PPD) into the inner-forearm. Results of the test are based on the diameter of the induration 48 to 72 hours following administration. While the TST is generally effective, results require several days to obtain and specificity is low in populations vaccinated with the TB-vaccine, BCG, due to overlapping antigens present in both the vaccine and PPD (57). These issues likely spurred the recent development of interferon gamma release assays (IGRA), which yield results within 24 hours and utilize antigens not found in BCG (58).

Treatment of individuals at risk for transitioning from LTBI to active TB disease is an important strategy in fighting the global TB epidemic. Treatment for LTBI typically includes some combination of self-administered isoniazid and/or rifampetine and is considered to be highly effective in decreasing the probability of progressing to active TB disease (59). The challenge in implementing treatment of LTBI is identification of when and in whom treatment should be initiated. The global population with LTBI includes an estimated two billion people, and it is unlikely that all of these individuals could or should be subjected to a lengthy course of antibiotics. Thus, an important step in treating LTBI will be identifying those individuals who are at risk of progressing to TB and in need of treatment. The ability to identify who is at risk for

transitioning from an infected and stable state to an infected but disease progressing state is critical for both the clinical management of the individual and for the prevention of TB transmission at the population level. Importantly, biomarkers capable of identifying these individuals at risk for progression have not yet been established, though early efforts to do so using genetics (60), immunological markers (61), and metabolomic profiles (62) are ongoing.

Role for Iron in TB

Evidence links iron with TB pathogenesis both from the perspective of the pathogen and the host. Upon infection, host immune recognition of *Mtb* induces a pro-inflammatory reaction that restricts iron access (63). Since host-acquired iron is a co-factor involved in vital *Mtb* cellular processes, *Mtb* responds by manufacturing siderophores, molecules capable of binding iron more strongly than host iron-storage proteins. Specifically, *Mtb* synthesizes two types of siderophores: lipophilic, cell-bound mycobactins and free carboxymycobactins, which work together to capture iron from a variety of host iron sources (including transferrin, lactoferrin and heme (64, 65)) and transfer it across the *Mtb* cell wall. This process has been shown to be essential for *Mtb* growth and virulence (66, 67) and capable of increasing iron availability in the mycobacterial phagosome almost 20-fold (68). Evidence highlighting the importance of siderophores to *Mtb* growth is consistent with studies examining the effect of modified iron status on *Mtb* growth. Decreasing iron availability (regardless of the mechanism) reduces *Mtb* growth, and addition of iron to *Mtb* almost always enhances growth (63). Similarly, genetics support the important role for iron in *Mtb* growth. *Mtb* mutants with defective genes involved in siderophore synthesis (66) and/or iron-utilization machinery (69) show reduced growth.

Examination of the role of iron in TB from the perspective of the host echoes its importance. Most strikingly, a pattern of altered host iron status characterized by high ferritin, low transferrin, and low hemoglobin has been identified as a risk factor for progression to TB among HIV-infected individuals (70). This is consistent with evidence indicating that high macrophage iron stores are linked to an increased likelihood of contracting *Mycobacterium spp.* infections (71) as well as studies suggesting that dietary iron overload is associated with an increased risk of developing pulmonary tuberculosis (PTB) (72) and dying from TB (73). It is also consistent with studies that have identified anemia is a risk factor for poorer TB outcomes (53, 54). From the genetic perspective, polymorphisms in the phagolysosomal iron transporter *SLC11A1* (NRAMP1) are associated with TB susceptibility (74, 75) and hereditary hemochromatosis, which leads to iron deficient macrophages, is thought to be protective against TB (76).

HIV/TB Co-Infection Prevalence, Pathogenesis and Treatment

The probability of developing and dying from TB is significantly higher in people infected with HIV. An estimated 13% of all new TB cases and 27% of deaths from TB in 2013 were in HIV-infected people (39).

Epidemiological evidence suggests that HIV alters the pathogenesis of TB. Among those infected with *Mtb*, people living with HIV are 12-20 times more likely to progress to active TB than those not living with HIV (77). The mechanisms explaining this altered pathogenesis, however, are not clear. Studies have shown that risk of TB increases with CD4+ T cell depletion, but also that a high risk of TB is present when CD4+ T cell counts are higher during the first year

following HIV seroconversion (78). It may be that TB-specific CD4+ T cells are depleted (79) or that HIV alters T-cell responses in a way that eases progression from LTBI to active TB. The latter is supported by a report that HIV-infected subjects that showed decreased responses to *Mtb* antigens linked with LTBI (80). Similar studies have also reported that T-cell responses to *Mtb* antigens are influenced by CD4+ T cell counts and ART exposure (81, 82).

Treatment of HIV/TB co-infection is inclusive of both ART and standard TB treatment. ART has been shown to reduce the likelihood of progression to TB (83) and anti-TB treatment is recommended upon diagnosis of TB (84). The World Health Organization (WHO) also recommends the use of Isoniazid Preventative Therapy (IPT) for all people living with HIV. IPT is considered safe, and significantly reduces the risk of progression from LTBI to active TB. The WHO considers IPT to be one its three priorities, along with intensified case finding and infection control, to reduce the impact of TB on people living with HIV.

Role for iron in HIV/TB Co-Infection

A small number of studies have specifically explored the relationship between iron homeostasis and TB in the context of HIV, with most focusing on associations at TB diagnosis or post TB-diagnosis clinical outcomes. These studies have linked both iron status to TB recurrence (85) and anemia to mortality in HIV/TB co-infection (86). At the current time, just one study has examined iron status prior to TB diagnosis, allowing for an investigation into factors associated with TB susceptibility. In this study, which was mentioned previously, a pattern of altered host iron homeostasis characterized by high ferritin, low transferrin, and low hemoglobin was identified as a risk factor for progression to TB among HIV-infected individuals (70).

Overall Research Objective

Given the evidence supporting a role for iron in the pathogenesis of HIV, HIV/TB co-infection, TB and anemia, the objectives of this research were to characterize changes in iron homeostasis and provide insight into the mechanisms governing iron homeostasis in these contexts. Additionally, we aimed to explore the potential clinical utility of monitoring iron homeostasis biomarkers at HIV diagnosis, prior to the development of TB disease and during TB therapy.

Specific Aims

- 1) To investigate hepcidin concentration as a risk factor for incident TB in HIV infection.
- 2) To investigate hepcidin concentration as a risk factor for mortality in HIV and explore the broader links between anemia, inflammation and iron homeostasis in HIV.
- 3) To investigate iron homeostasis biomarkers as risk factors for progression to active TB disease.
- 4) To evaluate anemia etiologies and characterize the evolution of iron homeostasis biomarkers during TB therapy.

Study Populations and Setting

The studies described in this document were conducted using archived plasma and serum samples from the Medical Research Council (MRC) HIV clinical cohort and the MRC TB case-contact platform, both of which were/are based in The Gambia. Briefly, the HIV clinical cohort was started in the late 1980s and included men and women from throughout The

Gambia who were recruited at all stages of HIV progression at a time when antiretrovirals (ARVs) were unavailable. The ongoing MRC TB case-contact (TBCC) research platform is designed to follow both household contacts of infectious TB cases and TB cases undergoing treatment (87). It includes primarily participants from the urban and semi-urban areas along The Gambian coast. Clinical data and plasma/serum samples were collected from both the HIV cohort and the TBCC participants at regular intervals along with demographic data.

The Gambia is a small country in West Africa geographically surrounded by Senegal except for a small stretch of Atlantic Ocean coastline. The majority of the population of almost two million people is concentrated in the urban and semi-urban areas along the coast. The population is primarily Muslim (90%) and includes a variety of ethnic groups (Mandinka, 42%; Fula, 18%, Wolof, 16%; Jola 10%; Serahuli 9%; other 4%; non-African, 1%) (88). The World Bank considers The Gambia to be a low-income country, with almost 50% of the population below the poverty line (89). HIV prevalence in The Gambia is estimated to be 1.3%, the 35th highest in the world (88). TB incidence in The Gambia is also among the highest in the world, at 173 cases/100,000 population (39). Other significant health risks in The Gambia include anemia (4), malaria, dengue fever, undernutrition, schistosomiasis, meningococcal meningitis and diarrhea.

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CHAPTER 2

Elevated hepcidin at HIV diagnosis is associated with incident tuberculosis in a retrospective cohort study*

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Abstract

Hepcidin inhibits ferroportin-mediated iron efflux leading to intracellular macrophage iron retention possibly favoring *Mycobacterium tuberculosis* iron acquisition and tuberculosis pathogenesis. Plasma hepcidin was measured at HIV diagnosis in a retrospective HIV-seroprevalent, antiretroviral-naïve African cohort to investigate the association with incident pulmonary and/or extrapulmonary tuberculosis. One hundred ninety-six participants were followed between August 5, 1992 to June 1, 2002, with 32 incident tuberculosis cases identified. Greater hepcidin was associated with significantly increased likelihood of tuberculosis after a median time to tuberculosis of six months. Elucidation of iron-related causal mechanisms and time-sensitive biomarkers that identify individual changes in tuberculosis risk are needed.

Introduction

Identifying tuberculosis (TB) risk factors is challenging due to variable disease latencies. Host-pathogen iron homeostasis has been previously implicated, and hepcidin, a small liver-derived peptide that promotes degradation of the intracellular inorganic iron exporter ferroportin, may also play a part (1). Expression is stimulated by pro-inflammatory cytokines resulting in ferroportin blockade of iron release from intestinal enterocytes and macrophages. Through its influence on host iron homeostasis, hepcidin may promote conditions favorable to *Mycobacterium tuberculosis* (Mtb). Thus, this study was designed to investigate the relationship between hepcidin and susceptibility to TB using a retrospective analysis of a longitudinal cohort.

Methods

From a historical HIV-seroprevalent cohort in The Gambia (described elsewhere (2)), 196 participants aged ≥ 18 years with a plasma sample obtained within three months of HIV diagnosis were included. To address temporality, plasma samples were obtained at least 28 days prior to an incident TB diagnosis and prevalent TB cases excluded. Provisional TB diagnoses were independently reexamined according to study-specific incident TB case definitions: confirmed pulmonary TB (PTB) was acid-fast bacilli in direct smear/culture from sputum/lavage; smear-negative PTB was strongly suggestive clinical symptoms and radiographic signs consistent with PTB; and/or confirmed extra-pulmonary TB (EPTB) was acid-fast bacilli in biopsy/aspirates of a lymph node; probable EPTB was strongly suggestive clinical features of EPTB. Ethical approval was granted by the Joint Gambian Government/MRC, The London School of Hygiene & Tropical Medicine and Cornell University.

Plasma hepcidin was quantified using a competitive EIA (Bachem, USA). Samples were assayed in duplicate and dilutions based on ferritin concentrations. Hepcidin concentrations were interpolated from 4-parameter logistic standard curves. The lower limit of detection (LoD) was interpolated at three standard deviations from the all plate mean OD450 readings (wells containing diluent in lieu of hepcidin standard or sample). Undiluted samples <LoD were imputed with LoD/2. Concentrations outside the linear region of the curve or with an intra-assay coefficient of variation >15% were re-assayed. As an indicator of inflammation, *alpha*₁-antichymotrypsin (ACT) was measured using a nephelometric assay (DakoCytomation Inc, UK).

Associations were assessed using Poisson regression (STATA/MP 11.1; College Station, TX) and expressed as incidence rate ratios (IRR) with incident TB (all TB) as the main outcome. Covariates considered *a priori* were gender and baseline ACT, absolute CD4 cell count, HIV-type, age and body mass index (BMI).

Results

Incident TB cases (n=32) were identified from 196 participants followed 496 person-years [median follow-up; interquartile range (IQR)=1.8 years; 1.3 to 2.3]. Median time to TB diagnosis was 0.5 years (IQR=0.3 to 1.1). Baseline differences (median, IQR; mean \pm standard deviation; frequency) between incident TB and non-cases were not statistically significant for hepcidin 22.1, 3.3 to 85.9 ng/ml; CD4 250, 91 to 502 cells/ μ l; age 34.4 \pm 10.3 years; Hb 10.5 \pm 2.3 g/dL; ACT 0.49 \pm 0.27 g/L; BMI 19.9 \pm 4.3 kg/m²; HIV-1 60.2% and female 55%.

A dose-response association, with a possible threshold effect, was observed between hepcidin and the likelihood of incident TB (Figure 1). The upper hepcidin quartile was associated with the greatest probability of developing TB with the upper quartile accounting for

>40% of all incident TB cases. Comparing the upper hepcidin quartile to the lower three quartiles combined revealed a two-fold increase in the incidence of TB [unadjusted incident rate ratio (IRR); 95% CI=2.05; 1.01 to 4.16]. The magnitude and direction of associations were consistent between the unadjusted and adjusted hepcidin models (Figure 2-top), however, only the model with hepcidin adjusted for HIV-type was statistically significant (adjusted-IRR; 95% CI=2.10; 1.03 to 4.26). As expected, *a priori* considered covariates representing greater immunosuppression or inflammation, lower BMI and HIV-1 were associated with non-statistically significant greater probability of TB in unadjusted models (Figure 2-bottom).

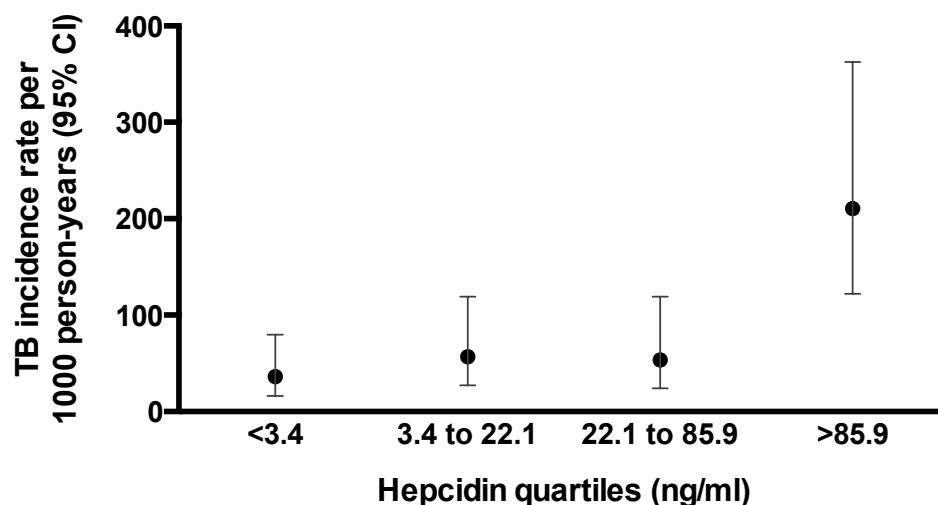


Figure 2.1 Incident tuberculosis classified by hepcidin quartiles at HIV diagnosis

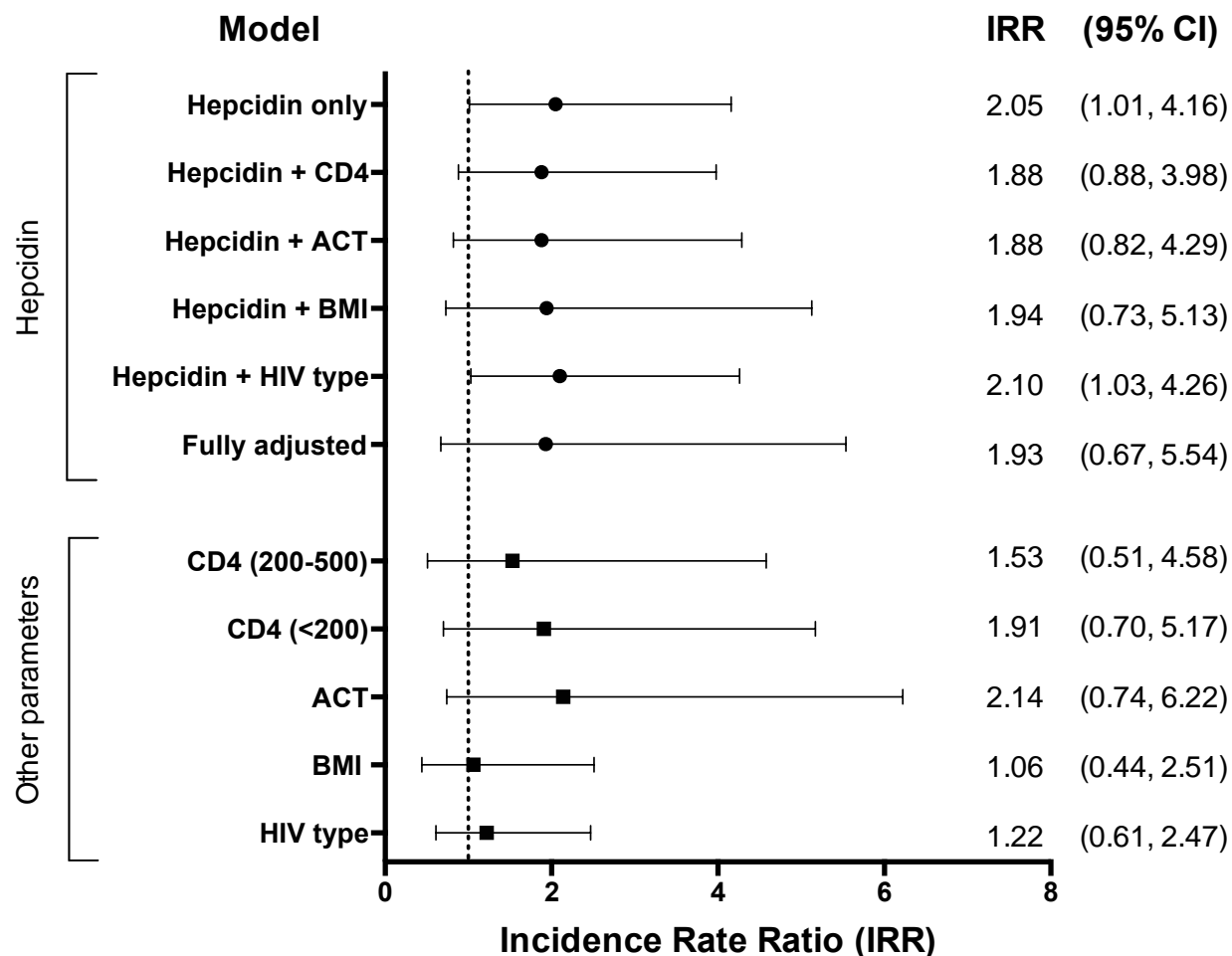


Figure 2.2 Plasma hepcidin at HIV diagnosis (top) and additional baseline risk factors (bottom) in relation to incident tuberculosis

IRR=Incident rate ratio; BMI=body mass index; ACT= α_1 -antichymotrypsin

Variables were continuous except for: hepcidin concentrations were classified in quartiles with the combined lower three quartiles as the referent; absolute CD4 cell counts were classified in clinical categories <200, 200-500 and >500 cells/ μ L as the referent; HIV type was classified as HIV-1 plus HIV-Dual or HIV-2 as the referent; BMI was classified as <18.5 kg/m² or \geq 18.5 kg/m² with as the referent. The fully adjusted model included HIV type, absolute CD4 cell count, ACT, age, gender and BMI. All models included 196 participants except for models with missing data for CD4 (182 participants included), BMI (151 participants included) and the fully adjusted model (145 participants included).

Discussion

A unique strength of this study is our ability to investigate the temporal association between hepcidin as a risk factor for TB susceptibility rather than TB prognosis. These data suggest that in HIV infection, higher hepcidin concentration is associated with a significantly greater risk of developing TB. A recent report from Indonesia indicated median hepcidin concentrations at cohort entry were also significantly higher among 45 incident TB cases compared to controls with HIV infection (3), however, this was evident only among cases diagnosed between 31 to 60 days after cohort enrollment. Using a different statistical analysis, our findings demonstrate that the window may extend further as baseline hepcidin was associated with incident TB diagnosed a median of six months after enrollment. While hepcidin is most likely part of a complex multi-parameter risk profile with the relevant time period remaining to be identified, together these findings suggest rising hepcidin may be a proxy biomarker for subclinical TB.

The mechanisms explaining the association between hepcidin and incident TB in HIV infection are unknown. Hepcidin appears to fit into a larger picture of inflammatory iron redistribution including hemoglobin, plasma iron, ferritin, soluble transferrin receptor and transferrin that has been previously reported by this group using a larger subset of these same cohort participants (4). Hepcidin has an important regulatory role in host iron homeostasis during infection, and pathogen iron homeostasis including both iron acquisition and storage influences TB pathogenesis (5-8). However, how elevated hepcidin modulates the host iron environment to favor *Mtb* is unknown. Interestingly, emerging evidence suggests that *Mtb* may actually manipulate host cellular hepcidin production (9) to create a pathogen iron-favorable

environment. If this is the case, expanding ongoing research investigating hepcidin antagonists (10) to consider hepcidin antagonists in the prevention of TB or augmentation of existing TB treatment may be warranted.

In summary, greater hepcidin concentrations at HIV diagnosis are associated with a greater probability of TB with HIV co-infection. Understanding the relationship between hepcidin, host-pathogen iron homeostasis, immune activation/inflammation and TB pathogenesis may guide evidence-based decisions in the clinical management of people at high risk of iron-deficiency anemia and TB, major health concerns in many regions of the developing world. Overall, this study contributes evidence needed to create a profile identifying who is at greatest risk of progressing to clinical TB, and when transition from an infected-stable state to an infected-progressing state occurs; obstacles critical to overcome to be able to improve individual TB management and population-level TB control.

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CHAPTER 3

Elevated hepcidin is part of a complex relationship that links mortality with iron homeostasis and anemia in men and women with HIV infection*

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Abstract

Background: Early and chronic inflammation is a hallmark of HIV infection, and inflammation is known to increase hepcidin expression. Consequently, hepcidin may be a key determinant of the iron homeostasis and anemia associated with poorer HIV prognoses.

Objective: To understand how hepcidin is related to anemia, iron homeostasis and inflammation at HIV diagnosis, and investigate associations between hepcidin and all-cause mortality in HIV infection.

Methods: In a retrospective cohort, baseline plasma hepcidin was measured by competitive enzyme immunoassay within three months of HIV diagnosis in 196 antiretroviral-naïve Gambians. Iron homeostasis (hemoglobin, plasma transferrin, ferritin, iron, soluble transferrin receptor) and inflammation (α_1 -antichymotrypsin) from the same aliquot were available, as were absolute CD4 cell count, age, gender, body mass index and HIV type.

Results: Anemia was common across the spectrum of immunosuppression (CD4>500 anemia=68%; 200 to 500=73%; <200=89%, $p=0.032$), and in men (81%) and women (76%). Increasing hepcidin was associated with iron homeostasis biomarkers (higher ferritin and lower transferrin, hemoglobin, soluble transfer receptor), inflammation (higher α_1 -antichymotrypsin) and key health indicators (lower CD4 or body mass index, advancing age, male gender) (p -values<0.001, except hemoglobin=0.021). Elevated hepcidin was associated with greater all-cause mortality in a dose-dependent manner (unadjusted Hazard Ratio middle vs. lowest tertile=1.95, 95%CI=1.22-3.10; upper vs. lowest: 3.02, 1.91-4.78). Principal component analysis identified two patterns comprised of hepcidin-ferritin-transferrin, with or without α_1 -antichymotrypsin, and iron-soluble transferrin receptor-hemoglobin that may distinguish

inflammation and erythropoiesis iron functions.

Conclusions: Elevated hepcidin is independently associated with greater mortality in HIV infection, and hepcidin is also part of a complex relationship linking iron homeostasis, anemia and HIV. Understanding mechanisms and the role of hepcidin modulation may further guide evidence-based interventions needed to counter detrimental iron homeostasis and anemia in HIV infection.

Introduction

Anemia and abnormal iron distribution are associated with increased morbidity and mortality in HIV infection (1-5). Nutritional causes of anemia including iron, folate and B12 deficiencies that are considerable in regions affected by poverty (6, 7). People living in these regions are frequently burdened by infectious diseases, and the associated chronic immune activation and inflammation they experience fuels anemia of inflammation (AI) (8). Since iron homeostasis is affected by immunological, infectious, clinical and nutritional contributors, it acts as a barometer of overall health status.

Early and chronic immune activation and inflammation is a hallmark of HIV infection. Under inflammatory conditions, dietary iron is blocked from enterocyte release, while circulating iron is redistributed into cellular storage locations that include macrophages. This affects iron delivery needed for erythropoiesis, and additional HIV-associated consequences including myelosuppression, impaired erythropoietin production and opportunistic infections further contribute to anemia in HIV infection (9, 10). These processes may explain why anemia is such a common hematological disorder before antiretroviral (ART) therapy is initiated (11). While ART reduces the prevalence of anemia for many people, a considerable proportion experience unresolved anemia or develop anemia following ART initiation (Table 3.1).

Hepcidin is a protein integrally involved in iron homeostasis and anemia through its interaction with the only known vertebrate cellular iron exporter, ferroportin (reviewed in (12)). Inflammation or excess iron stores lead to up-regulation of hepcidin expression and synthesis, while erythropoiesis and hypoxia are negative regulators (13). Hepcidin inhibits iron efflux from duodenal enterocytes and increases intracellular iron retention in iron-recycling macrophages.

Recent reports indicate hepcidin may influence multiple HIV outcomes since higher hepcidin concentrations were associated with increased *in vitro* HIV-1 transcription (14), as well as greater immunosuppression and tuberculosis among HIV-positive Indonesians (15). These outcomes have been previously linked with other iron-related biomarkers (16-18), suggesting that hepcidin is just one contributor to the complex iron biology in HIV infection. Despite the importance of hepcidin and the iron regulation associated with anemia, and the link between hepcidin and inflammation that has been demonstrated in other clinical conditions (19-21), the role of hepcidin in the context of HIV infection and HIV-related anemia remains undetermined. Consequently, this study was designed to characterize hepcidin concentrations at HIV diagnosis in relation to anemia, iron homeostasis and inflammation, and to investigate hepcidin as an independent risk factor for mortality in HIV infection in a region where both infectious and nutritional contributors are common.

Table 3.1. Anemia in HIV infection in Africa

| Study | Participant characteristics | Definition of anemia | Anemia at ART initiation ^a | Pre-ART anemia risk factors | Persistent or incident anemia after ART initiation ^b | Post-ART anemia risk factors |
|--|-----------------------------------|---|---|--|--|---|
| Johannessen A, Naman E, Gundersen SG, Bruun JN. <i>BMC Infect Dis</i> 2011;11:190. | Adults in rural Northern Tanzania | Mild anemia: Hb<13 g/dL for males Hb<12 g/dL for females Moderate anemia: 8-10 g/dL Severe anemia: <8 g/dL | Total: 77.4% (649/838) Mild: 32.1% Moderate: (28.4%) Severe: (16.9%) 41.9% of anemia was microcytic | Female Advanced WHO Stage Low BMI Tuberculosis Microcytosis Hypochromia | Total: 38.2% (39/102) After 12 months on ART Mild: (29.4%) Moderate: (6.9%) Severe: (2%) | Low MCV at ART initiation, Zidovudine-containing initial regimen |
| Moh R, Danel C, Sorho S, et al. <i>Antivir Ther</i> 2005;10:615-624. | Adults in urban Cote d'Ivoire | Hb<10.5 g/dL | 31% | | 28% after 3 Months on HAART; 17% after 6 months on HAART | Low baseline Hb |
| Forna F, Moore D, Mermin J, et al. <i>J Int Assoc Physicians AIDS Care</i> 2009;8:128-138. | Adults in rural Uganda | Hb≤10 g/dL | | | 25.6% (279/1089) After (media=34.2; IQR=10.1-36.8) months (median) on ART | Anemia at baseline >12 months of d4T before switch zidovudine |
| Kiragga AN, Castelnuovo B, Nakanjako D, Manabe YC. <i>J Int AIDS Soc</i> 2010;13:42. | Adults in Uganda | Hb≤9.5 g/dL Hb≤8 g/dL for severe anemia | 15% (821/5494) 5.4% (296/5494) for <u>severe</u> anemia | For <u>severe</u> anemia: Female Advanced HIV stage | 4% after 6 months of ART for <u>severe</u> anemia | Baseline severe anemia Incident tuberculosis Low MCV |
| Rawat R, McCoy SI, Kadiyala S. <i>J Acquir</i> | Adults in Uganda | Hb<10 g/dL | 7.7% (55/716) | | | |

| | | | | | | |
|--|--|--|--|--|---|---|
| <i>Immune Defic Syndr</i> 2013,62:246-253. | | | | | | |
| Mugisha JO, Shafer LA, Van der Paal L, <i>et al.</i> <i>Trop Med Int Health</i> 2008,13:788-794 | Adults in rural Uganda | Hb<12 g/dL for males Hb<11 g/dL for females | 18.9% (49/259) | Female Advanced WHO stage Low CD4 Wasting | | |
| Jaganath D, Walker AS, Ssali F, <i>et al.</i> <i>AIDS Res Hum Retroviruses</i> 2014. | Children, reproductive age adults and older adults in Uganda and Zimbabwe | Adults: Hb<12 g/dL for males Hb<11 g/dL for females Children: Hb<10.5 g/dL (ages 5- 11) Hb<11 g/dL (ages 12- 14) | 43% (1527/3580) 30% of anemia was microcytic | | 13% after approx. 10 months (96 weeks) on ART 12% of anemia was microcytic | Older adults Female Low BMI Microcytosis |
| Takuva S, Maskew M, Brennan AT, <i>et al.</i> <i>J Trop Med</i> 2013,2013:162950. | Adults in Johannesburg, SA | Hb<10 g/dL | 25.8% | Female Younger Advanced WHO stage Tuberculosis | Incident anemia=16.7/1000 person-years between 3 and 6 months after starting ART | Female gender Low BMI Advanced WHO Stage Low CD4 AZT use |
| Owirededu WK, Quaye L, Amidu N, Addai-Mensah O. <i>Afr Health Sci</i> 2011,11:2-15. | Adults in the Upper-East region (rural) of Ghana | Hb≤10.5 g/dL | 63% | | 46% after 3+ months on HAART | |
| Ferede G, Wondimeneh Y. <i>BMC Hematol</i> 2013,13:8. | Ethiopia Adults | Hb<13 g/dL for males Hb<12 g/dL for females | 35% (138/400) | Female gender Low CD4 | | |
| Adane A, Desta K, Bezabih A, <i>et al.</i> <i>Ethiop Med J</i> 2012,50:13-21. | Adults in urban Ethiopia | | 52.6% | Female gender | 37.4% after 4 months on ART | Female gender |
| Gedefaw L, Yemane T, Sahlemariam Z, Yilma D. | Children, adults and | <u>Adults:</u> Hb<13 g/dL for males | 29.9% | Opportunistic infections | 16.2% after 3+ months on HAART | HAART regimen |

| | | | | | | |
|--|------------------------------------|---|--------------------|----------------------------|--|-------------------|
| <i>PLoS One</i> 2013;8:e72202 | older adults in Southwest Ethiopia | Hb<12 g/dL for females <u>Children:</u> Hb<12 g/dL (ages 12 to <15y) Hb<11.5 g/dL (ages 5 to <12y) Hb<11 g/dL (ages 0.5 to <5y) | | Low CD4 Rural residence | | Duration of HAART |
| Tadesse K, Haile F, Hiruy N. <i>PLoS One</i> 2014;9:e87392. | Adults in Northern Ethiopia | <11 g/dL | 43% (223/520) | | | |
| Munyazesa E, Emile I, Mutimura E, <i>et al. BMJ open</i> 2012;2. | Adult women in Rwanda | <12 g/dL | 24.7% (165/669) | Low BMI Low CD4 | | |

ART=antiretroviral therapy; BMI=body mass index; HAART=highly active antiretroviral therapy; Hb=hemoglobin; MCV= mean corpuscular volume with microcytic anemia=MCV defined as <80 fL; WHO=World Health Organization

^a Inclusive of mild, moderate and severe anemia for ART-naïve individuals initiating ART or HAART and not initiating ART or HAART(due to lack of availability and/or standard of care when the studies were conducted)

^b Inclusive of both incident and persistent anemia in individuals taking ART or HAART for at least 3 months, specific definitions of ART and HAART can be found in the references articles

Methods

Study setting

Participants were recruited from a larger prospective cohort study (22) and sub-study (16, 23) of HIV-positive Gambians. Eligibility criteria included ≥ 18 years and an existing baseline plasma aliquot archived within three months of first HIV diagnosis. Cohort participants were provided free clinical care at the Medical Research Council Unit in The Gambia in accordance with national HIV guidelines in effect at the time of participation, including cotrimoxazole prophylaxis and symptom management. Viral load analysis was unavailable for routine monitoring at the time of sample collection. Ethical approval for this study was granted by the Joint Gambian Government / Medical Research Council Ethical Review Committee, The London School of Hygiene & Tropical Medicine and Cornell University.

Follow-up and all-cause mortality ascertainment

Participants (n=196) enrolled between August 5th, 1992 and August 22, 2001, with follow-up observation continuing until June 1, 2002. All-cause mortality was ascertained at study clinic visits scheduled every three months. Participants who missed appointments were considered lost-to-follow-up if their mortality status could not be determined by medical records or at home visits. Participants were censored on the last date they were known with certainty to be alive defined as the last date of clinic attendance or the last date seen by a fieldworker or the end of the study observation period. As common in this region of Africa, cause-specific mortality was unavailable since autopsies, including verbal autopsies (24), were not routine.

Hepcidin, anemia, iron homeostasis and inflammation

All iron assays were performed using a single heparinized plasma aliquot that was stored at -80°C. This single plasma sample was thawed, and then subdivided into multiple microvials and refrozen at -80°C. A single microvial was obtained from the freezer and thawed for a second time until batch processing of each analyte. The period between refreezing and hepcidin analysis was longer than for other analytes. While the long-term stability of hepcidin is unknown due to the relatively recent commercial availability of this assay, the presence of four disulfide bonds in the folded structure suggests that the peptide should maintain long-term stability (25). Assay quality controls provided by the assay manufacturer were used and performed within manufacturer-defined limits and pooled plasma obtained from a local blood bank was used to assess quality assurance. Hepcidin concentrations were measured in duplicate using a competitive enzyme immunoassay (Bachem, Peninsula Laboratories, San Carlos, CA) according to the manufacturer's protocol (26, 27). Samples were diluted based on known ferritin concentrations in supplied standard diluent (peptide-cleared human serum) and analyzed using a 9-point 2-fold serial dilution (maximum concentration, 25 ng/mL) standard curve. Hepcidin concentrations were interpolated from 4-parameter logistic standard curves generated by readerfit (www.readerfit.com). The lower limit of detection of 0.40 ng/mL was interpolated at three standard deviations from the all plate mean (e.g. wells containing diluent in lieu of hepcidin standard or sample). The 11 undiluted samples with limits of detection <0.40 ng/mL were imputed with a value of limit of detection/2. Samples with concentrations outside the linear region of the curve, or those with an intra-assay coefficient of variation >15%, were reanalyzed using appropriate dilutions.

Soluble transferrin receptor (sTfR) (R&D Systems, Abingdon, UK) and ferritin (Immuno-biological Laboratories, Hamburg, Germany) were measured by ELISA, and limit of detections were <3 and >80 nmol/L, and <2.5 and >1000 µg/L, respectively; samples with values outside of these ranges were imputed with the limit of detection value. Plasma iron was assessed using an endpoint assay (ABX Diagnostics, Shefford, UK) and transferrin by turbidimetry (ABX Diagnostics, Shefford, UK). Hb concentrations were measured with a hematology analyzer using routine procedures in the clinical laboratory (Coulter MD II, Coulter Corporation, USA), and values obtained from study databases. Alpha₁-antichymotrypsin (ACT), as an indicator of inflammation, was measured using a nephelometric assay (DakoCytomation Inc, Ely, UK).

Statistics

STATA/MP 11.1 (College Station, TX) was used for statistical analyses. Data transformation included categorization of Hb, ferritin, sTfR and iron according to clinical reference ranges. Inspection of raw data suggested using only the clinical reference range would obscure meaningful transferrin associations, and transferrin was reported according to both clinical reference limits and tertiles. In the absence of an established clinical reference range for hepcidin, hepcidin was classified into tertiles. ACT is primarily a research analyte and a clinical reference range was not provided by the manufacturer or recommended by others (28), however, for this statistical analysis, a frequently cited cut-off range was used (29).

Scatterplots and boxplots were used to graphically present bivariate associations between hepcidin and Hb, iron homeostasis and inflammation biomarkers, as well as the *a priori* considered potential covariates [body mass index (BMI), age, absolute CD4 cell count, gender, HIV type]. Pearson correlation coefficients, Wilcoxon rank sum, ANOVA, Student's *t*-

test, Spearman or χ^2 were used to test these associations, with a p -value <0.05 considered statistically significant. A Kaplan–Meier survival curve was used to graphically represent the probability of survival from HIV diagnosis according to hepcidin tertiles and compared using the Log-rank test. Univariable and multivariable Cox regression models with all-cause mortality as the main outcome were analyzed.

Principal Component Analysis (PCA) was chosen as a variable reduction technique given the potential redundancy (e.g. correlation) in the measured iron homeostasis (transferrin, ferritin, iron, Hb, sTfR) and inflammatory (ACT) variables. Essentially, the goal of PCA was to reduce the number of observed variables to allow concentration on only those that principally contribute to variation in a meaningful way. This was done by statistically identifying groups of variables that “travel together” in components (e.g. patterns), while at the same time, disregarding those variables that only contribute in a negligible way. Identified patterns represent distinct groups that are uncorrelated from other patterns. Decisions regarding which components were meaningful to retain was based on the *a priori* criterion of eigenvalue-one (patterns with eigenvalues >1 were considered to contribute principally).

PCA was conducted with iron and inflammatory variables categorized in tertiles and also as continuous variables; since similar results were obtained, only tertile models reported. After identifying principal patterns of iron and inflammation groupings, principal component (PC)-scores were calculated for each individual. These scores represent a linear algebraic equation accounting for the weighting of each observed variable. PC-scores were modelled as a continuous variable and analyzed in unadjusted and adjusted Cox regression. An iron homeostasis index was also calculated for each participant based on the PCA-identified pattern

groupings. The iron homeostasis index conceptually represents the magnitude of iron deviation and combined impact on mortality. It was calculated using the observed concentrations measured for each iron and inflammatory variables classified into tertiles. A value of 1 was assigned to the lowest hazard ratio associated with each biomarker. In these data, the lowest hazard ratios were associated with the lowest tertile of hepcidin, ferritin, sTfR, ACT and the highest tertiles of transferrin, iron and hemoglobin. All intermediate tertiles were assigned a value of 2; and all remaining tertiles were assigned a value of 3 (e.g. those associated with the greatest hazard ratios). Values were summed for each individual providing an overall iron homeostasis index specific for each individual and each PCA-identified pattern. The iron homeostasis index was modelled as a single continuous variable and analyzed using unadjusted and adjusted Cox regression.

Results

Subjects

Characteristics of the 196 cohort participants are summarized in Table 3.2. During the 10-year cohort follow-up period, 64% of participants died (n=125), 19% (n=37) were censored at the last date with known certainty to be alive and 17% (n=34) were alive at study closure. Participants censored prior to death or study closure were more likely to have greater absolute CD4 cell counts like commonly observed in resource-restricted regions without universal ART access, and in this study, were also more likely to be younger and female.

Hepcidin and association with iron homeostasis, anemia and inflammation in HIV

Hepcidin was positively correlated with ferritin and ACT and inversely correlated with Hb, transferrin and sTfR, but not plasma iron (Figure 3.1). Higher hepcidin concentrations were

correlated with lower absolute CD4 cell counts or BMI and advancing age, but there was no apparent association with HIV type (Figure 3.2). While males had higher hepcidin than women (median, IQR hepcidin: men=52.4, 16.7 to 96.9 vs. women=11.9, 2.4 to 58.6 ng/m; $p<0.001$), males were also experiencing greater immunosuppression at HIV diagnosis (median, IQR CD4 cells/ μ l: men=191,136 to 255 vs. women=292, 218 to 399; $p<0.001$).

Table 3.2 summarizes participant characteristics according to anemia classification. Anemia was associated with a lower mean BMI and more advanced immunosuppression as expected, but anemia was also common across the spectrum of immunosuppression (CD4>500 anemia=68%; 200 to 500=73%; <200=89%, $p=0.032$), and in both men and women (frequency of anemia in men=81%, women=76%; mean \pm SD Hb concentration in men= 10.3 ± 2.6 , women= 10.7 ± 2.1 g/L). Anemia was associated with a greater degree of inflammation as indicated by greater ACT concentrations (anemia vs no-anemia: 0.43 vs 0.32 g/L; $p=0.003$), as well as an inflammation-induced iron redistribution profile typified by comparatively greater hepcidin and ferritin and lower transferrin and iron concentrations (p -values <0.09). While sTfR concentrations were higher in the anemic group suggesting the presence of iron deficiency or co-existing iron deficiency plus AI, the sTfR/ \log_{10} ferritin ratio was not significantly different between those with or without anemia.

Hepcidin and mortality in HIV

Figure 3.3 demonstrates a dose-response relationship between greater hepcidin at HIV diagnosis and increasing probability of all-cause mortality. This relationship is further supported in unadjusted Cox regression analysis, however, the adjusted association was attenuated and not statistically significant (Table 3.3). Like hepcidin, all other iron homeostasis

and inflammatory associations were attenuated in regression models adjusting for HIV type, age, gender, BMI, immunosuppression at HIV diagnosis, with or without additional adjustment for ACT. Anemia, very elevated ferritin and the lowest transferrin tertile remained statistically significant in adjusted analyses.

Principal component analysis of iron homeostasis and inflammation in HIV

Using PCA (Table 3.4), two unique patterns were identified that accounted for considerable cumulative variance (68%) in a PCA model with all iron homeostasis variables (PCA1). The first pattern (PCA1.1) was greatly influenced by the trio of hepcidin-ferritin-transferrin that together explained 43% of the total variance. The second pattern (PCA1.2) was primarily influenced by plasma iron-sTfR-Hb, explaining 25% of the variance. Notably, the identified patterns were driven by non-overlapping principal components (e.g. the observed variables did not contribute in a meaningful way to both patterns). In a second model (PCA2) that included all iron homeostasis variables plus ACT, similar grouping patterns were identified. In this model that accounted for 66% of the variance, the first pattern (PCA2.1) explaining 45% of the variance was dominated by the quartet of hepcidin-ferritin-transferrin-ACT, and the second pattern (PCA2.2) explaining 21% of the variance was again primarily influenced by plasma iron-sTfR-Hb. In both PCA models, the principal component eigenvectors (e.g. the relative weighting attributed to each of the variables within a specified pattern) between hepcidin, ferritin, transferrin with or without ACT were of comparative magnitude, while iron was comparatively more influential than sTfR or Hb.

Mortality associations using predicted PC-scores, as well as an iron homeostasis index based on PCA-identified patterns, are presented in Table 3.4. PC-scores derived from

weightings assigned to variables in patterns PCA1.1 (hepcidin-ferritin-transferrin) or PCA2.2 (hepcidin-ferritin-transferrin-ACT) were significantly associated with mortality in unadjusted and adjusted analysis, whereas the PC-scores derived from variables that grouped together in patterns PCA1.2 or 2.2 (both included iron-sTfR-Hb) were not. Analysis of the iron homeostasis index demonstrated that as the degree of iron deviation typified by the combination of elevated hepcidin, elevated ferritin and lower transferrin (PCA1.1) or elevated hepcidin, elevated ferritin, lower transferrin and elevated ACT (PCA2.1) increased, so did the mortality hazard ratio in unadjusted and adjusted models, although adjusted associations were attenuated. Like the PC-scores modeling, the iron homeostasis index reflecting lower iron, higher sTfR and lower Hb was associated with increased likelihood of mortality. In both the PC-score and iron homeostasis index models, the associations with hepcidin, ferritin, transferrin, with or without ACT, were comparatively stronger than for iron, sTfR and Hb.

Table 3.2. Characteristics of cohort participants at HIV diagnosis

| | Overall^a n=196 | Anemia^b n=110 | No Anemia n=30 | P-value^c |
|---|-------------------------------------|------------------------------------|--------------------------|----------------------------|
| Cohort person-years | 1.8 (0.6 to 4.1) | 1.2 (0.5 to 3.3) | 4.1 (1.7 to 7.9) | <0.001 |
| Age (years) | 34.3 ± 9.8 | 33.0 ± 8.8 | 34.6 ± 10.1 | 0.42 |
| Female | 55% | 53% | 60% | 0.48 |
| HIV status | | | | 0.65 |
| HIV-1 | 60% | 65% | 60% | |
| HIV-2 | 39% | 35% | 40% | |
| HIV-dual ^d | 1% | 0% | 0% | |
| Absolute CD4 (cells/μl) (n=182) | 250 (92 to 503) | 233 (77 to 498) | 408 (243 to 699) | 0.003 |
| Body mass index (kg/m ²) (n=178) | 19.7 ± 4.0 | 19.2 ± 3.5 | 21.6 ± 5.1 | 0.005 |
| Body mass index <18.5 kg/m ² , % | 37% | 33% | 33% | |
| α ₁ -antichymotrypsin (g/L) | 0.41 (0.31 to 0.58) | 0.43 (0.39 to 0.46) | 0.32 (0.28 to 0.36) | 0.003 |
| Hepcidin (ng/mL) | 22.1 (3.3 to 85.9) | 32.2 (2.2 to 88.7) | 6.8 (2.1 to 53.3) | 0.06 |
| Ferritin (μg/L) | 161 (37 to 534) | 158 (36 to 643) | 90 (28 to 191) | 0.09 |
| Transferrin (g/L) | 1.80 ± 0.64 | 1.75 ± 0.67 | 2.00 ± 0.50 | 0.06 |
| Iron (μmol/L) | 9.0 (6.4 to 14.4) | 8.5 (6.4 to 12.6) | 15.0 (10.0 to 18.8) | <0.001 |
| Soluble transferrin receptor (nmol/L) | 24.4 (17.0 to 35.5) | 25.3 (21.5 to 28.2) | 19.8 (16.6 to 23.0) | 0.020 |
| Soluble transferrin receptor/log ₁₀ ferritin | 11.1 (7.1 to 19.0) | 11.3 (7.3 to 18.7) | 10.4 (6.5 to 24.1) | 0.55 |
| Hemoglobin (g/L) (n=140) | 10.5 ± 2.3 | 9.6 ± 1.7 | 13.8 ± 1.1 | <0.001 |

IQR, interquartile range; n, sample size; SD, standard deviation.

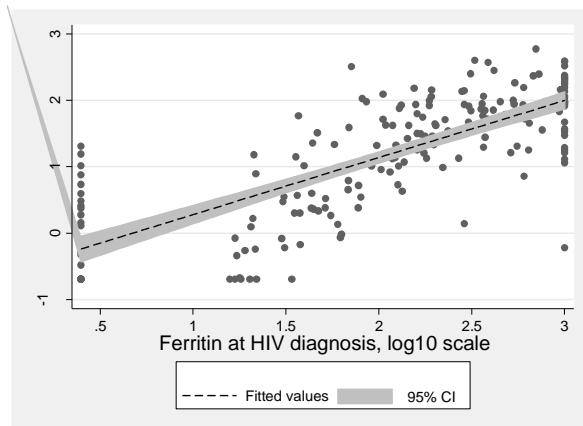
^aFor normally distributed continuous variables, data presented as mean \pm standard deviation; non-normally distributed continuous variables presented as median and interquartile range; categorical variables presented as frequency.

^bAnemia was defined according to WHO definition of Hb male <13 ; female <12 g/d (30). Hb was available for 140 participants, therefore the anemia and no anemia participants totals 140.

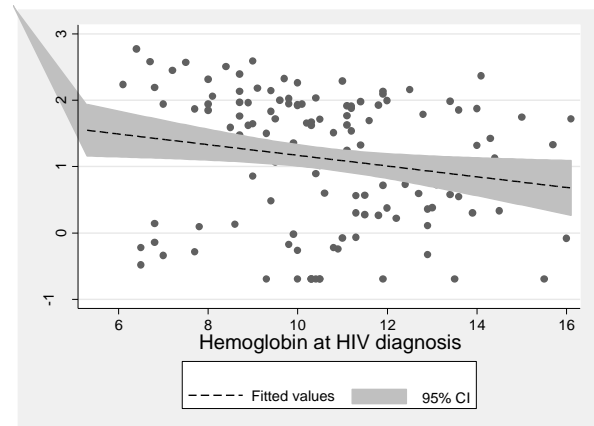
^cP-values calculating by comparing group means (Student's *t*-test for normal distribution, continuous and Wilcoxon rank-sum test for non-normal, continuous) and or frequencies (χ^2 test) between anemic vs. non-anemic groups.

^dGiven the small number of HIV-dual diagnoses, HIV-2 was combined with the HIV-1 group for subsequent analyses.

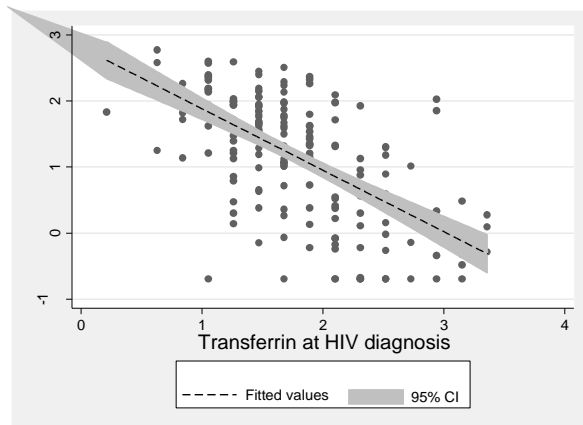
Figure 3.1. Scatterplot and correlation coefficients (r) with corresponding p -values between circulating hepcidin concentration at HIV diagnosis (ng/ml, \log_{10} scale, y-axis) of HIV Clinical Cohort participants, The Gambia (1992 to 2001) and: (A) plasma ferritin ($\mu\text{g/L}$, \log_{10} scale), (B) hemoglobin (g/L), (C) plasma transferrin (g/L), (D) plasma soluble transferrin receptor (nmol/L, \log_{10} scale), (E) plasma iron ($\mu\text{mol/L}$, \log_{10} scale) (F) age (years), (G) α_1 -antichymotrypsin (g/L, \log_{10} scale), (H) body mass index (kg/m^2).



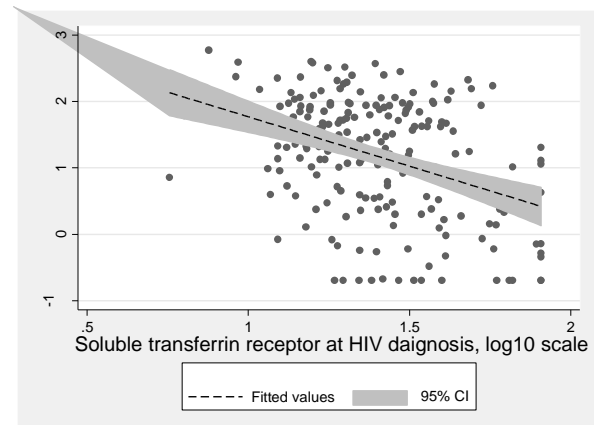
A. $r=0.76$; $p < 0.001$



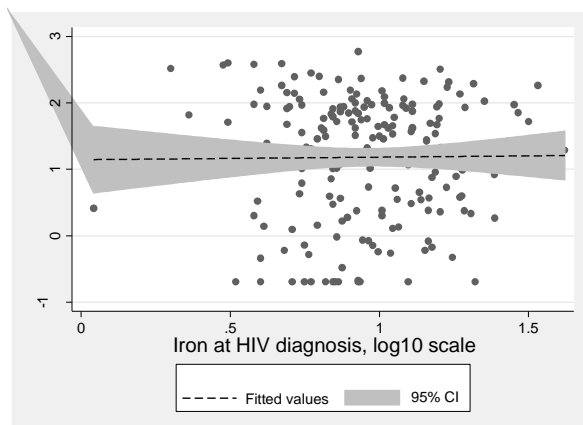
B. $r=-0.20$; $p < 0.021$



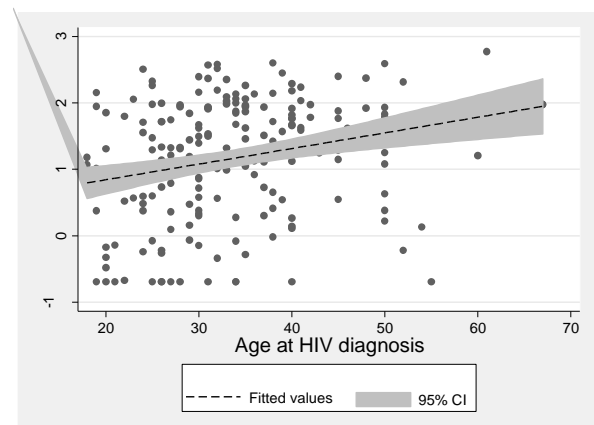
C. $r=-0.61$; $p < 0.001$



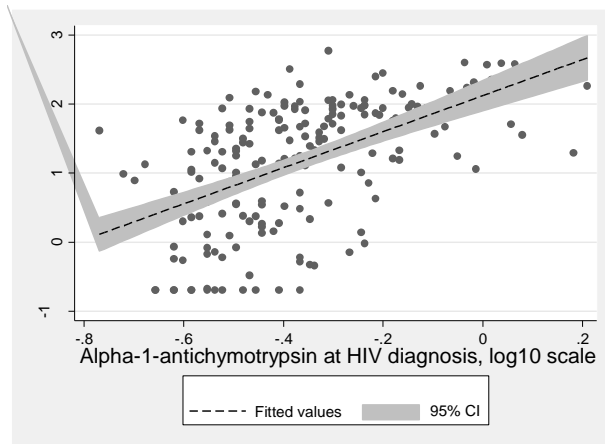
D. $r=-0.38$; $p < 0.001$



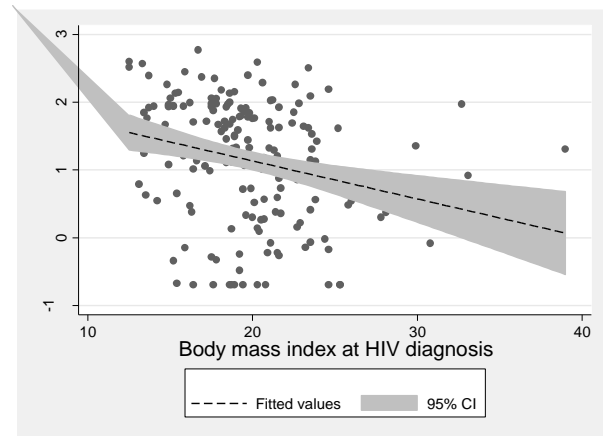
E. $r=-0.01$; $p=0.88$



F. $r=0.27$; $p < 0.001$



G. $r=0.57$; $p < 0.001$



H. $r=-0.26$; $p=0.001$

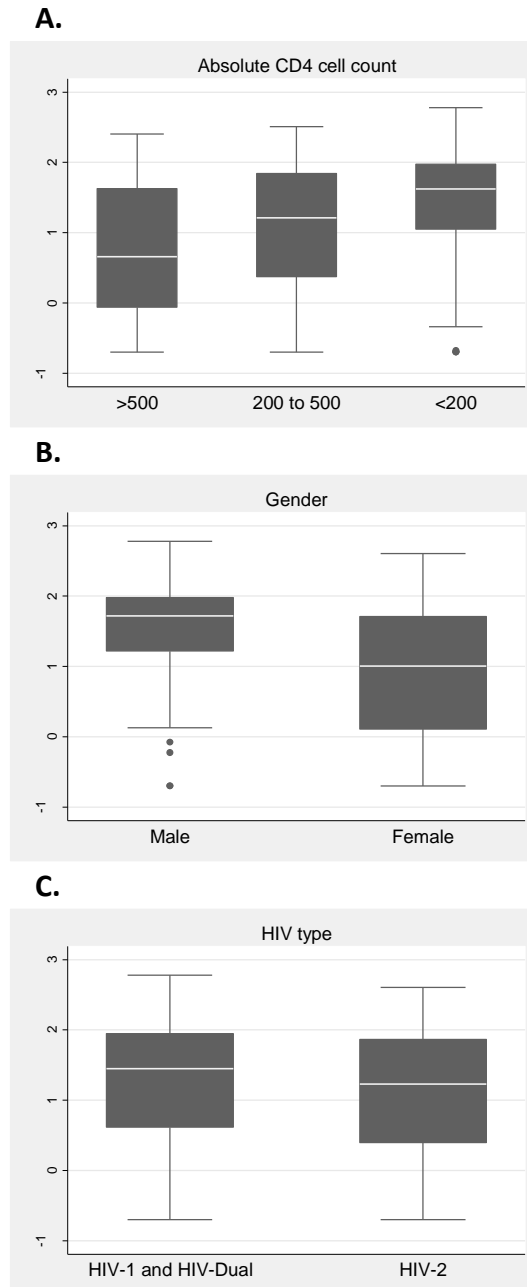


Figure 3.2. Box-plots of circulating hepcidin concentrations (ng/mL; \log_{10} scale) at HIV diagnosis of HIV Clinical Cohort participants, The Gambia (1992 to 2001) categorized according to: (A) absolute CD4 cell count (cells/ μ l): <200 (median: 62.8; IQR=18.5 to 175.9), 200-500 (median: 46.5; IQR=3.9 to 87.3), >500 (median: 20.2; IQR=1.7 to 44.8) ($p<0.001$); (B) gender: male (median: 52.4; IQR=16.7 to 96.9), female (median: 11.9; IQR=2.4 to 58.6) ($p<0.001$); and (C) HIV type: HIV-1 plus HIV-Dual (median: 41.8; IQR=6.2 to 93.7), HIV-2 (median: 19.4; IQR=3.1 to 75.3). HIV-Dual was grouped with HIV-1 due to small numbers ($n=2$) ($p=0.40$). Differences in mean \log_{10} -transformed hepcidin concentrations were evaluated using Student's t -test or ANOVA.

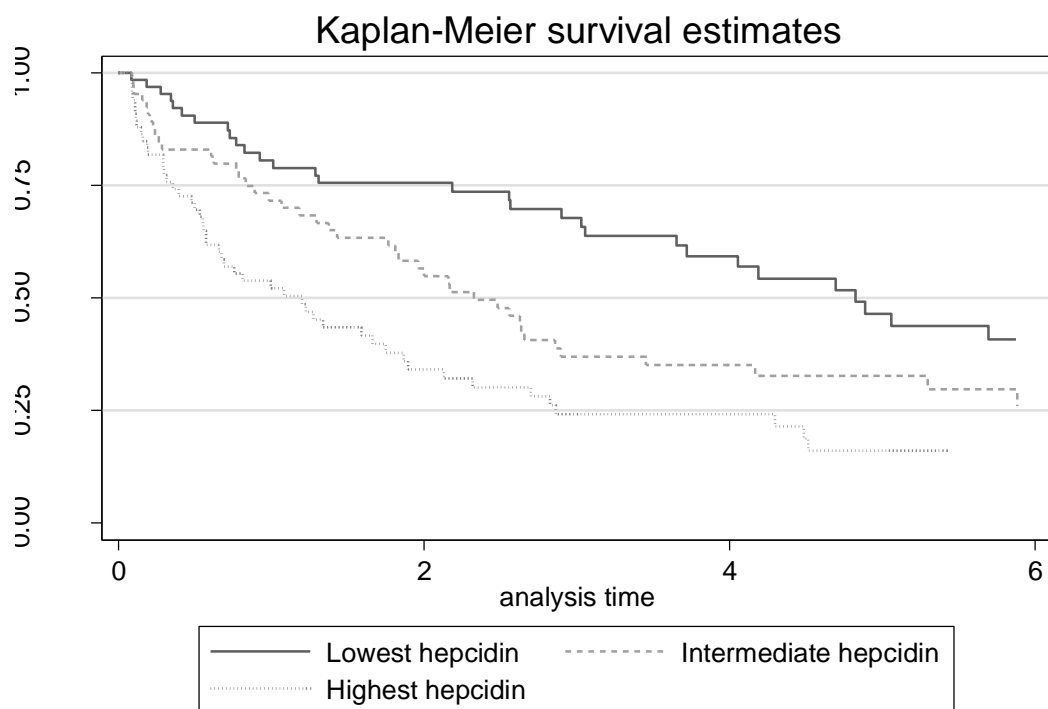


Figure 3.3. Kaplan-Meier survival curves according to hepcidin tertiles at HIV diagnosis
 Lowest hepcidin tertile ≤ 7.8 ng/mL; intermediate > 7.8 to < 57.6 ; highest ≥ 57.6 ng/ml

Table 3.3. Cox regression models^a of hepcidin, iron homeostasis and inflammation and associations with all-cause mortality in HIV infection

| Biomarker | Concentration at HIV diagnosis | Clinical cutoff or tertile limits ^b | Univariate models, HR (95% CI) ^c | Adjusted models, HR (95% CI) ^{c, d} | Adjusted models plus α_1 -antichymotrypsin, HR (95% CI) ^{c, e} |
|---------------------------------------|--------------------------------|--|---|--|--|
| Hepcidin (ng/ml) | Lowest tertile | ≤7.8 | Reference | Reference | Reference |
| | Intermediate | >7.8 to <57.6 | 1.95 (1.22 to 3.10) | 0.96 (0.56 to 1.63) | 0.97 (0.56 to 1.67) |
| | Highest | ≥57.6 | 3.02 (1.91 to 4.78) | 1.07 (0.61 to 1.87) | 1.11 (0.59 to 2.08) |
| Ferritin (μg/L) | Lower than normal | <12 | 0.62 (0.31 to 1.21) | 0.61 (0.29 to 1.25) | 0.58 (0.28 to 1.21) |
| | Normal | See ^f | Reference | Reference | Reference |
| | Elevated | See ^f | 2.10 (1.37 to 3.23) | 1.57 (0.97 to 2.51) | 1.90 (1.14 to 3.18) |
| | Very elevated | >1000 | 3.91 (2.44 to 6.28) | 2.09 (1.19 to 3.67) | 2.78 (1.49 to 5.17) |
| Transferrin (g/L) | Highest tertile | ≥1.89 | Reference | Reference | Reference |
| | Intermediate | >1.47 to <1.89 | 2.42 (1.37 to 4.24) | 0.78 (0.39 to 1.57) | 0.79 (0.40 to 1.58) |
| | Lowest | ≤1.47 | 4.36 (2.85 to 6.66) | 1.92 (1.12 to 3.31) | 2.13 (1.21 to 3.75) |
| Transferrin (g/L) | Lower than normal | <2.0 | 2.81 (1.81 to 4.37) | 1.02 (0.57 to 1.84) | 1.03 (0.57 to 1.88) |
| | Normal ^g | 2.0 to 3.6 | Reference | Reference | Reference |
| Iron (μmol/L) | Lower than normal | <20 | 1.24 (0.87 to 1.77) | 1.02 (0.68 to 1.53) | 1.02 (0.68 to 1.54) |
| | Normal ^h | 20 to 55 | Reference | Reference (n=168) | Reference (n=168) |
| | Elevated | >55 | 0.93 (0.22 to 3.82) | 0.91 (0.60 to 1.37) | 0.78 (0.18 to 3.27) |
| Soluble transferrin receptor (nmol/L) | Lower than normal | <10.6 | 0.87 (0.35 to 2.16) | 0.52 (0.2 to 1.35) | 0.52 (0.20 to 1.35) |
| | Normal ⁱ | 10.6 to 29.9 ^h | Reference | Reference | Reference |
| | Elevated | >29.9 | 1.05 (0.73 to 1.53) | 0.91 (0.60 to 1.38) | 0.90 (0.58 to 1.38) |
| Hemoglobin (g/L) | Anemic | See footnote ^j | 3.26 (1.75 to 6.07) | 2.75 (1.31 to 5.76) | 2.72 (1.29 to 5.72) |
| | Non-anemic | See footnote ^j | Reference | Reference | Reference |
| α_1 -antichymotrypsin (g/L) | Lower than normal | <0.2 | 0.74 (0.10 to 5.33) | 0.95 (0.12 to 7.38) | NA |
| | Normal ^k | 0.2 to 0.6 | Reference | Reference | NA |
| | Elevated | >0.6 | 1.89 (1.27 to 2.80) | 1.00 (0.60 to 1.66) | NA |

CI, confidence interval; HR, Cox regression hazard ratio; NA, not applicable.

^aUnadjusted models sample size=196, except for 140 for hemoglobin models; adjusted models have 168, except for 119 for hemoglobin models due to missing data.

^bThere is no established clinical reference range for hepcidin, and distribution of raw transferrin data indicated tertile classification was informative and therefore both tertiles and clinical-cutoffs included for transferrin.

^cThe category with the lowest risk of mortality served as the reference category.

^dAdjusted for HIV type (HIV-1 plus HIV-dual or HIV-2), age, gender, body mass index, and absolute CD4 cell count (>500, 200 to 500, < 200 cells/ μ L) at HIV diagnosis. Gender was not included in regression models where gender used to establish clinical cut-offs (anemia, ferritin); and age not included when age used to classify ferritin categories.

^eAdjusted for ACT (continuous) plus all variables in footnote^c.

^fFerritin normal reference ranges: 18 to 44 years: men 12 to 200 μ g/L, women 12 to 150 μ g/L; \geq 45 years: men 12 to 300 μ g/L, women 12 to 200 μ g/L (31).

^gTransferrin normal reference range: 2.0 to 3.6 g/L (32); no participants had above normal transferrin concentrations.

^hIron normal reference range: 20 to 55 μ mol/L (32)

ⁱSoluble transferrin receptor normal reference range: (33)

^jAnemia: Hb <13 g/L for men; Hb <12 g/L for women (30).

^kACT normal reference range: 0.2 to 0.6 g/L (32). A normal reference range was not provided by the assay manufacturer, and given assay method sensitivity and inconsistencies regarding the existence of a “normal” reference range for ACT, the normal limits should be interpreted with these considerations (28).

Table 3.4. Principal components analyses of iron homeostasis and inflammatory variables and Cox regression analyses of all-cause mortality^a

| PCA model | Grouping pattern identified by PCA | Total variance explained by pattern | Principal Components contributing to grouping pattern | Principal Component Eigen-vector ^b | Principal Component-Score ^b , unadjusted Hazard Ratios (95% CI) | Principal Component-Score ^c , adjusted ^d Hazard Ratios (95% CI) | Iron Dysregulation Index ^e , unadjusted Hazard Ratios (95% CI) | Iron Dysregulation Index ^e , adjusted ^d Hazard Ratios (95% CI) |
|-------------------|------------------------------------|-------------------------------------|---|---|--|---|---|--|
| PCA1 ^g | Pattern 1 (PCA1.1) | 43.4% | Hepcidin | 0.54 | 1.72 (1.49 to 1.99) | 1.31 (1.07 to 1.61) | 1.37 (1.26 to 1.50) | 1.13 (1.00 to 1.27) |
| | | | Ferritin | 0.54 | | | | |
| | | | Transferrin | 0.53 | | | | |
| | Pattern 2 (PCA1.2) | 24.7% | Iron | 0.63 | 1.08 (0.93 to 1.27) | 1.00 (0.83 to 1.20) | 1.15 (1.03 to 1.29) | 1.08 (0.80 to 1.45) |
| | | | sTfR | 0.54 | | | | |
| | | | Hemoglobin | 0.49 | | | | |
| PCA2 ^h | Pattern 1 (PCA2.1) | 44.8% | Hepcidin | 0.48 | 1.64 (1.44 to 1.87) | 1.26 (1.05 to 1.51) | 1.28 (1.19 to 1.37) | 1.08 (0.99 to 1.89) |
| | | | Ferritin | 0.48 | | | | |
| | | | Transferrin | 0.46 | | | | |
| | | | ACT | 0.45 | | | | |
| | Pattern 2 (PCA2.2) | 21.3% | Iron | 0.61 | 1.06 (0.90 to 1.24) | 0.98 (0.82 to 1.18) | 1.15 ^f (1.03 to 1.29) | 1.08 ^f (0.80 to 1.45) |
| | | | sTfR | 0.56 | | | | |
| | | | Hemoglobin | 0.46 | | | | |

ACT, α_1 -antichymotrypsin; CI, confidence interval; PCA, principal component analysis; sTfR, soluble transferrin receptor.

^aUnadjusted models sample size=196, except for 140 for hemoglobin models; adjusted models have 168, except for 119 for hemoglobin models due to missing data.

^bOrdered by relative loading or impact of eigenvector (e.g. weighting of individual variable, where more important principal components are assigned greater weights); only variables with meaningful eigenvectors (e.g. >0.40) are presented.

^cCox regression using calculated Principal Component-Scores for each participant where the scores represent a linear algebraic combination of all variables included in the PCA model and weighted by eigenvectors.

^dAdjusted for HIV type (HIV-1 plus HIV-dual or HIV-2), age, gender, body mass index, absolute CD4 cell count (>500, 200 to 500, < 200 cells/ μ L) at HIV diagnosis.

^eThe iron dysregulation index represents the combined impact on mortality of the variables in the principal component analysis-identified patterns. The observed concentrations measured for each iron and inflammatory variables were classified into tertiles whereby a value of 1 was assigned to the lowest hazard ratio (refer to Table 3.3). In these data, the lowest mortality was associated with the lowest tertile of hepcidin, ferritin, ACT and sTfR and the highest tertiles of transferrin, iron and hemoglobin. All intermediate tertiles were assigned a value of 2; and all remaining tertiles were assigned a value of 3 (e.g. those associated with the greatest hazard ratio for mortality in Table 3.3). Values were summed for each individual to represent an overall iron dysregulation index specific for each pattern. The iron dysregulation index was modelled as a single continuous variable using unadjusted and adjusted Cox regression.

^fThe index for PCA1.2 and PCA2.2 is equivalent because the same pattern of principal components (e.g. iron-sTfR-hemoglobin) was identified in both PCA models whether ACT was included or not

^g6-dimensional model including all iron homeostasis variables entered in tertiles

^h7-dimensional model including all iron homeostasis variables plus ACT entered in tertiles

Discussion

This study characterizes hepcidin at the time of HIV diagnosis in relation to iron homeostasis, inflammation and all-cause mortality in men and women with HIV infection. The findings demonstrate that hepcidin is integrally linked with the complex iron distribution that is associated with inflammation in HIV infection, a key cause of AI. This study also provides the first indication that elevated hepcidin is a risk factor associated with mortality in HIV infection using unadjusted regression, though it is unclear whether the mechanism is mediated through its association with negative causes and/or consequences of inflammation, maladaptive iron distribution or a reason yet to be identified.

While data regarding hepcidin in HIV infection remains limited, the findings from this study support those of an Indonesian study reporting serum hepcidin was positively correlated with ferritin, and inversely correlated with Hb and absolute CD4 cell counts (15). Wisaksana et al. (15) also observed that elevated hepcidin at study entry was associated with an increased probability of starting tuberculosis treatment within one to two months. Increases in macrophage iron stored in ferritin due to elevated hepcidin expression in an already immunocompromised host may alter the host-pathogen competition for iron and increase susceptibility to macrophage-tropic pathogens like *Mycobacterium tuberculosis*. While increased susceptibility to opportunistic infections like tuberculosis may explain in part the poorer mortality prognosis associated with elevated hepcidin in the current study (34), the unavailability of cause-specific mortality data prevents testing this hypothesis with these data. Additional possible mechanisms linking elevated hepcidin and increased mortality are presented in Figure 3.4. The inverse association between hepcidin and absolute CD4 cells

observed in this study and others (15) suggests that elevated hepcidin may be attributable to advanced disease stage and corresponding elevated levels of inflammation. While increased hepcidin expression may be a consequence of inflammation, higher systemic hepcidin and the resulting transfer of iron from the bloodstream into macrophages may also contribute to HIV progression via enhanced HIV propagation and destruction of CD4 cells. In an *in vitro* study, iron export by ferroportin in the absence of hepcidin was associated with decreased HIV-1 transcription (14). Adding hepcidin counteracted the iron efflux leading to increased intracellular iron and altered HIV production in CD4 cells and macrophages.

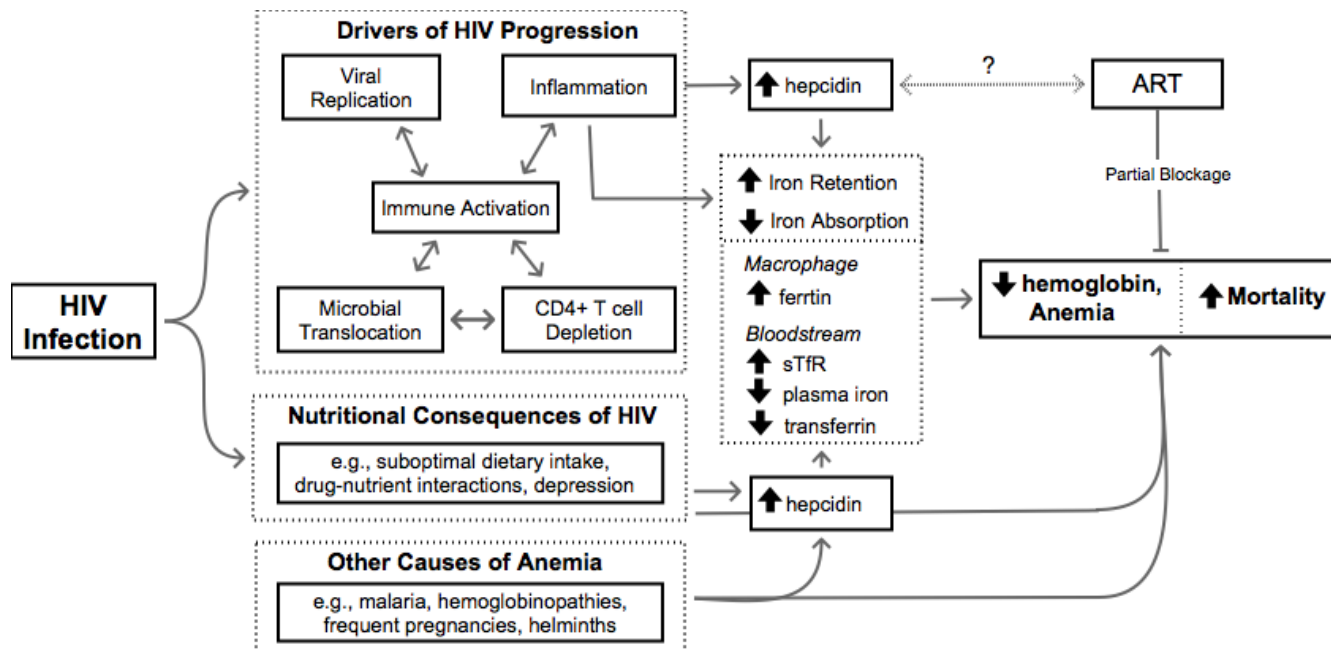


Figure 3.4. Pathways to HIV-associated anemia

A complex combination of interdependent factors including viral replication, microbial translocation, CD4 cell depletion, and chronic immune activation has been proposed as the driving force behind HIV disease progression (35). Production of pro-inflammatory cytokines (including IL-6) appears to both contribute to and also be dependent on factors driving disease progression, and in turn, stimulates hepcidin production and triggers the acute phase response. As a result, there is an increase in hepcidin-mediated ferroportin degradation and characteristic shift in iron from the bloodstream to the macrophage that is characterized by decreased plasma iron, decreased hemoglobin, decreased transferrin, increased sTfR and increased ferritin ultimately resulting in HIV-associated anemia. While ART is known to resolve some (but not all) HIV-associated anemia, its role in the proposed pathway is currently unknown. Alternatively, HIV-associated anemia may be the result of nutritional consequences of HIV and/or other (non-HIV-related) causes of anemia. Arrows indicate connected entities that are part of a pathway whereby solid arrows represent established pathways and dotted arrows remain hypothetical; lines ending without arrows indicate inhibition or blockage.

sTfR=soluble transferrin receptor; ART=antiretroviral therapy

Hepcidin expression is regulated by inflammation (upregulation), erythropoiesis and hypoxia (downregulation) and hepcidin concentrations are correlated with iron homeostasis biomarkers (e.g. ferritin, transferrin), although the relative hierarchy and biological importance of each under normal and clinical circumstances remains to be established (26, 36-38). By using statistical techniques designed to reduce redundancy of multiple correlated factors, further insight into the iron and infection relationship is possible. In this study, PCA revealed two distinct patterns, each dominated by a different group of variables. Given the specific variables statistically assigned to each pattern, it is possible to conceptually speculate that the first pattern is associated with inflammation (hepcidin-ferritin-transferrin, with or without ACT) and the second with erythropoiesis (iron-sTfR-Hb). While both patterns may be influencing aspects of anemia and iron homeostasis, the regression analyses suggests that the inflammatory-associated iron homeostasis pattern may be of greater relative importance in HIV. An understanding of the possible hierarchical nature of factors mediating hepcidin expression has not been elucidated for any infection. Early observations by Jonker and colleagues (37) suggest they are complex. In a study of severely anemic Malawian children living in a region with high malaria and generalized infectious disease burdens, low hepcidin concentrations were observed. Since infection-induced inflammation was likely contributing to increased hepcidin expression, the very low hepcidin concentrations seems counterintuitive. They speculated that under conditions of severe anemia-associated hypoxia and resulting increased erythropoiesis, infection-associated inflammatory signals that usually upregulate hepcidin may be overridden or down-regulated.

At this time, guidelines for anemia intervention in global settings include screening for malaria, treating helminth infection and providing iron/folate supplements; however, self-treatment using iron supplementation is common (39). This study provides support that elevated hepcidin is associated with anemia, and given the link with inflammation; screening to distinguish the type of anemia (e.g. AI and/or IDA) would help to select the most appropriate anemia intervention. Evaluation of Hb alone can determine neither the likely cause of anemia nor the best timing of interventions. Hepcidin concentrations have been shown to predict non-responsiveness to oral iron therapy (40), and in combination with other iron biomarkers such as ferritin and/or transferrin, hepcidin may guide in whom and when iron-based anemia interventions may be ineffective (27, 41, 42). Many hepcidin interventions are in development including hepcidin antagonists mediating effects through inhibiting hepcidin production (e.g. anti-inflammatories like anti-interleukin-6/interleukin-6R, anti-tumor necrosis factor- α), neutralizing hepcidin peptides (e.g. anti-calins, anti-hepcidin monoclonal antibodies) or interfering with hepcidin–ferroportin binding (e.g. thiol modifiers, anti-ferroportin monoclonal antibodies) (43, 44). While lowering hepcidin may be of benefit for iron homeostasis related to anemia, it could also negatively alter the host-pathogen competition for iron (37). Iron supplementation during active infection may overcome the universal iron sequestration mechanism mediated by the innate immune response. If this occurs while simultaneously intervening to lower hepcidin concentrations, a flood of iron dumped into the periphery could negatively alter the host-pathogen competition for iron (45), making infection control a prerequisite to iron-based anemia interventions.

The role of ART in mitigating hepcidin and iron homeostasis mechanisms remains to be elucidated (Figure 3.4), and the study is limited by the use of biological archives collected prior to widespread ART usage. While generalizability is limited to similar populations, the study findings have broad relevance as many people in developing countries present for HIV diagnosis with baseline characteristics similar to those in this study, others are diagnosed and lost to follow-up before ART is initiated and some drop-out of medical follow-up after ART initiation. For these groups, and as a reference for future studies seeking to identify and treat the subgroup of ART-treated individuals with residual or incident anemia (Table 3.1), the current data is important. Medication usage (ongoing or newly initiated) around the time individual plasma samples was unknown, and should be considered for future studies. Although these findings point to the importance of hepcidin in HIV infection, a portion of the statistical inferences were based on data-derived tertile categorizations of hepcidin and further research to identify clinically relevant cut-off points is needed. Lastly, while elevated hepcidin was associated with mortality in unadjusted regression models, the association was not statistically significant in models adjusted for a number of known mortality covariates (e.g. CD4, BMI, gender, age). Further studies may help to clarify the independent hepcidin association.

In summary, hepcidin is a piece of the complex and dynamic relationship linking HIV-associated anemia, iron homeostasis, inflammation and mortality in HIV infection. Higher hepcidin concentrations at HIV diagnosis are associated with a greater likelihood of mortality in men and women, and understanding how hepcidin evolves and influences iron homeostasis throughout early and chronic HIV infection is needed. This is especially important as many people with HIV suffer from anemia before ART initiation, and since ART may not fully resolve inflammation or

anemia. Overall, this study provides additional insight for the development of effective evidence-based decisions to prevent and manage HIV-associated anemia and maladaptive iron homeostasis occurring at all stages of HIV infection.

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CHAPTER 4

Iron homeostasis and progression to pulmonary tuberculosis disease among household contacts*

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Abstract

Early identification of individuals at risk for progressing to active tuberculosis (TB) disease may limit new transmission and improve clinical outcomes. Evidence indicates altered iron homeostasis may identify those at greater risk of disease progression in HIV co-infection. We aimed to investigate iron homeostasis biomarkers as risk factors for progression to TB. Archived plasma samples were analyzed from household contacts of pulmonary TB index cases in The Gambia. Contacts were classified as asymptomatic non-progressors (n=17) or TB-progressors (n=10), which included two HIV-infected participants. Iron homeostasis (hemoglobin, ferritin, hepcidin, soluble transferrin receptor, transferrin) was assessed in all contacts at study recruitment. Plasma was collected a median of 910 days prior to TB diagnosis. Low transferrin around the time of known exposure to infectious TB was a disease progression risk factor for all TB-progressors (Poisson incidence rate ratio: 0.55; 95% CI:0.35-0.89). Iron homeostasis also differed between early and delayed TB-progressors, with higher ferritin and hepcidin concentrations observed among early TB-progressors (mean ferritin 50.2 vs. 26.2 ng/ml; $P=0.027$; mean hepcidin 37.7 vs. 5.6 ng/ml; $P=0.036$). Iron homeostasis is associated with progression to TB among household contacts. Further studies are needed to elucidate mechanisms and determine the clinical utility of monitoring iron homeostasis biomarkers.

Introduction

Progression from uninfected or latent tuberculosis infection to clinical tuberculosis (TB) disease is frequently asymptomatic or associated with only mild symptoms during the first few months of disease (1). This can significantly delay diagnosis and treatment, resulting in higher TB mortality (2-4) and ongoing transmission (5, 6). Indeed, treatment delays are considered an important contributor to the approximately 1.5 million deaths and 9.0 million new TB cases reported each year (7, 8). Current approaches to reduce treatment delays include improved TB diagnostics (9) and active case finding strategies (10). While these approaches are helpful (11-13), identification of individuals at risk of disease progression could lead to further improvements.

Altered host iron status has been previously identified as a risk factor for progression to TB among HIV-infected individuals (14, 15), and a number of studies have indicated that *Mycobacterium tuberculosis* (*Mtb*) iron acquisition plays an important role in TB pathogenesis (16-20). A complex and intricate host-pathogen iron competition begins early in most infections. In TB, immune recognition of *Mtb* by the human host induces a pro-inflammatory reaction that restricts *Mtb* iron access (16). In response, *Mtb* manufactures siderophores, molecules capable of binding iron more strongly than host iron-storage proteins [reviewed in (21)]. Siderophore biosynthesis has been shown to be essential for *Mtb* growth and virulence (17, 19), suggesting that the success of *Mtb* in iron-scarce environments is due in part to its ability to acquire host iron. Despite the toxicity associated with iron excess, *Mtb* appears to also thrive when iron availability is increased (16). High macrophage iron stores have been linked to an increased likelihood of contracting *Mycobacterium spp.* infections (22), and dietary

iron overload has been associated with an increased risk of developing pulmonary TB (PTB) (23) or dying from TB (24).

Only a minority of exposed or infected people ever progress to clinical TB disease during their lifetimes. Among those that do progress, it typically occurs after a long clinical latency period. This presents considerable challenges to studying TB susceptibility risk factors since biomarkers of TB susceptibility need to be collected prior to the initiation of disease pathogenesis. As a result, biomarker studies require the prospective follow-up of large numbers of contacts of known infectious TB cases for a period of several years. To overcome this obstacle, this case-cohort study was designed to investigate iron homeostasis biomarkers as risk factors for progression to TB using archived plasma samples that were obtained as part of a larger TB case-contact study.

Methods

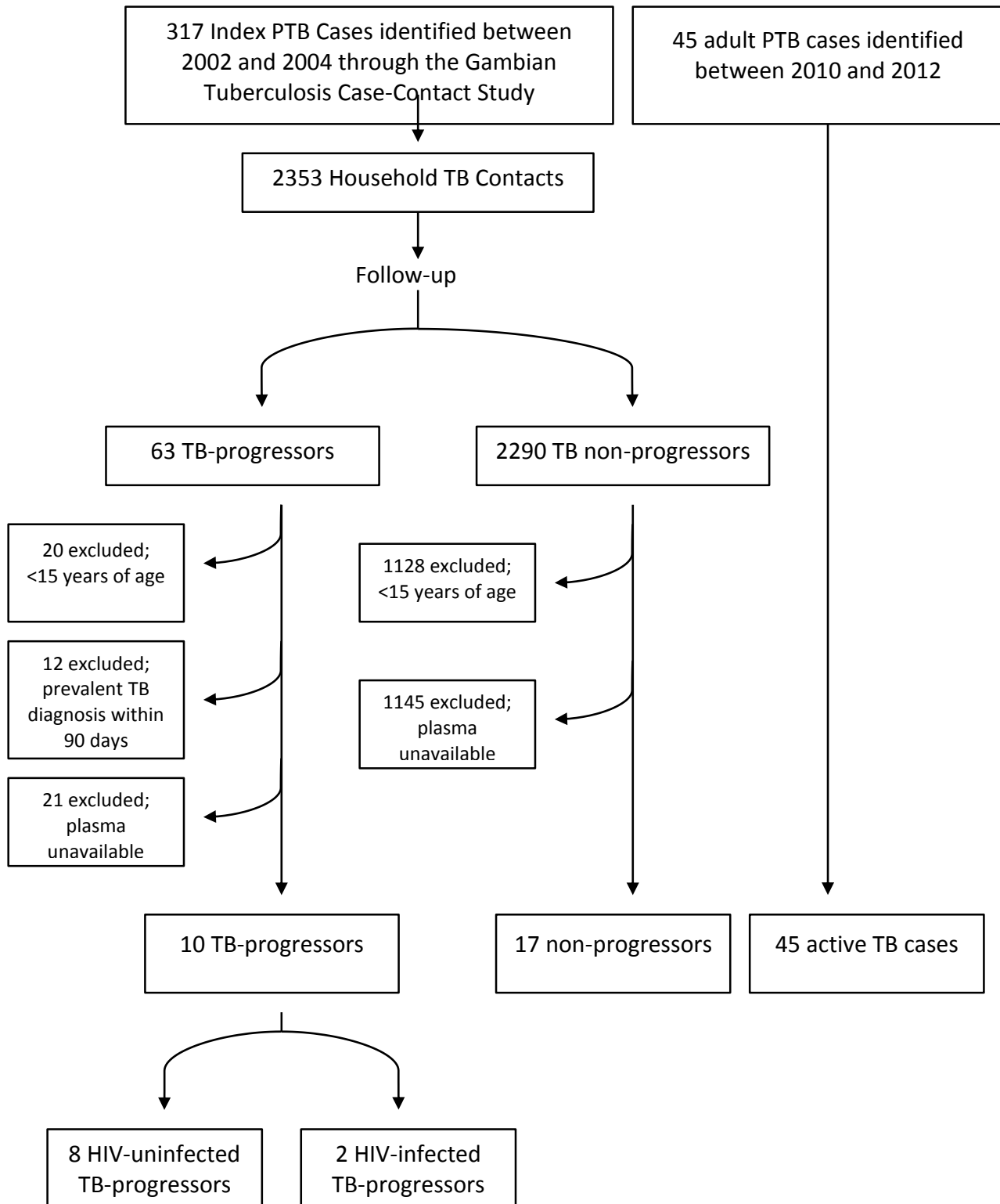
Study participants and definitions

Cryopreserved plasma and data from household contacts of PTB index cases recruited to the ongoing TB case-contact (TBCC) platform at the Medical Research Council Unit in The Gambia were used. The TBCC is a unique research platform designed to identify TB disease susceptibility risk factors in a large cohort of household contacts and has been described in detail elsewhere (25). Household contacts of index PTB cases were eligible to participate in the current case-cohort study if ≥ 15 years of age, maintained a shared residence with an index PTB case for ≥ 3 months prior to index case disease diagnosis and had a sufficient plasma sample archive for iron homeostasis analysis. All eligible TB-progressors cases identified within the larger cohort were included, as well as a random selection of non-progressors. In addition, TB-

progressors and non-progressors were compared with a third group of culture-confirmed active PTB cases at TB diagnosis (Figure 4.1). Ethical approval for the TBCC platform was granted by The Gambian Government/Medical Research Council Unit joint ethics committees, and additionally, for this case-cohort study by Cornell University.

All participants had a Tuberculin Skin Test (TST) (2 tuberculin units of Purified Protein Derivative RT23, Statens Serum Institute, Denmark) and underwent a clinical evaluation that included a comorbidity assessment with TB-specific clinical questions. Household contacts with symptoms consistent with TB were classified as “TB-progressors” if they developed active TB ≥ 90 days after study enrollment or were classified as “non-progressors” if they did not have evidence of TB throughout the follow-up period (Figure 4.1). TB-progressors were further categorized as “early TB-progressors” if they progressed to TB within two years of study entry or “delayed TB-progressors” if they developed TB after two years of follow-up.

Figure 4.1. Study Flowchart



TB=tuberculosis; PTB=pulmonary tuberculosis

Clinical and laboratory data

Enrollment characteristics included data on age, gender, body mass index (BMI), HIV-seropositivity, hemoglobin (Hb), white blood cell count and mean corpuscular volume (MCV). Plasma ferritin (Immuno-biological Laboratories, Germany), soluble transferrin receptor (sTfR) (R&D Systems, UK) and transferrin (Cygnus Technologies, USA) were measured by ELISA. Hepcidin was measured using a competitive enzyme immunoassay (Bachem, USA). All assays were optimized for use with plasma, and test samples, standards and controls were assayed in duplicate and concentrations interpolated from 4-parameter logistic standard curves (log/log curves for sTfR). **All standard curves were generated using SoftmaxPro 6 (Molecular Devices, USA).** Samples with an intra-assay coefficient of variation >15%, were re-assayed. Lower limits of detection for all assays, with the exception of hepcidin, were defined by the manufacturer. For hepcidin, the limit of detection (0.02 ng/ml) was interpolated at three standard deviations from the all plate mean (e.g. wells that contained diluent in lieu of hepcidin standard or sample).

Statistical Analysis

Median values of biomarkers and clinical measurements with non-normal distributions were compared using the Mann-Whitney *U*-test. Unpaired Student's *t* tests were similarly used for normally distributed biomarkers and measurements as well as comparisons of small samples. Chi-square tests were used to compare frequency distributions. Since median and/or mean concentrations of ferritin, sTfR and hemoglobin fell within normal clinical reference ranges, and a reference range for hepcidin has not yet been defined, iron homeostasis biomarker concentrations were standardized and modeled continuously. Univariate associations between standardized iron biomarkers and the binary outcome of "TB progression"

or “no TB progression” were assessed using Poisson regression with robust variance estimates. Poisson regression is considered an alternative to log-binomial regression when outcomes are common and odds ratios cannot be interpreted as relative risks (26). Given the mixed HIV-status of the TB-progressor group, all analyses were run in parallel with two groups of TB-progressors: one inclusive of the two HIV-seropositive individuals, the other excluding them. Examination of iron homeostasis biomarker concentrations (hemoglobin, ferritin, hepcidin, soluble transferrin receptor, transferrin) and clinical characteristics (BMI, MCV, white blood cell) suggested that HIV-infected TB-progressors were not statistically or clinically different from HIV-negative TB-progressors in measured risk factors. However, since the absolute CD4 cell counts were unknown and because HIV infection is known to be a strong risk factor for TB progression, the results of both the HIV-inclusive and HIV-exclusive analyses are presented. Statistical analyses were performed using STATA 13.0 (College Station, TX USA) and Prism 6.0 (La Jolla, CA USA).

Results

Demographic and clinical characteristics of the TBCC platform, the case-cohort subsample drawn from the TBCC platform and the active TB cases are summarized in Table 4.1. Overall, the case-cohort subsample was representative of the larger TBCC platform as participants were not significantly different based on age, gender, BMI, white blood cell and MCV. The median time to progression for all case-cohort TB-progressors was 910 days [interquartile range (IQR): 470 to 1337 days, n=10], and slightly longer at 1047 days (IQR: 611 to 1426, n=8) among TB-progressors who were HIV-negative. Early case-cohort TB progressors were diagnosed at approximately one year following study entry, and this did not differ

according to HIV-seropositivity, while delayed progressors were diagnosed at a median of 3.2 years. Known TB risk factors like age, gender and HIV status were not statistically different between TB-progressors, non-progressors or active TB cases. While BMI did not differ between TB-progressors and non-progressors, TB cases had significantly lower BMI and MCV and higher white blood cells. Additionally, significant differences were not detected in the comparison between TB-progressors when grouped with or without the two HIV-seropositive TB-progressors.

Table 4.1. Enrollment characteristics of age-eligible TB-progressors and non-progressors from the Medical Research Council TB Case-Contact (TBCC) platform (n=1193), TBCC subjects with available plasma samples (n=27) and among active tuberculosis cases prior to treatment initiation (n=45)

| | TB case-contact (TBCC) platform (e.g. source cohort) | | Case-cohort subsample of TBCC platform | | | Active TB cases, prior to treatment initiation |
|--------------------------------------|---|--------------------------|--|---|--|--|
| | Non-progressors (n=1162) | TB-progressors (n=31) | Non-progressors (n=17) | TB-progressors (HIV+ and HIV-) (n=10) | TB-progressors (HIV- only) (n=8) | n=45 |
| Age, years | 25 (20 to 36) | 26 (21 to 42) | 28 (20 to 39) | 27 (19 to 30) | 26 (19 to 28) | 29 (23 to 38) |
| Female, % | 58% (671/1162) | 55% (17/31) | 47% (8/17) | 50% (5/10) | 38%(3/8) | 38% (17/45) |
| HIV-infected, % | 4% (39/1051) | 13% (4/31) | 0% (0/17) | 20% (2/10) | 0% (0/8) | 4% (2/45) |
| Time to TB diagnosis (days) | | | | | | |
| All TB-progressors | N/A | 718 (436 to 1169) | N/A | 910 (470 to 1337) | 1047 (611 to 1426) | N/A |
| Early TB-progressors | N/A | 437 (151 to 607) | N/A | 386 (167 to 677) | 352 (150 to 553) | N/A |
| Delayed TB-progressors | N/A | 1169 (1035 to 1401) | N/A | 1175 (973 to 1535) | 1175 (973 to 1535) | N/A |
| Body mass index (kg/m ²) | 21.6 ± 4.6 (n= 1153) | 20.3 ± 3.3 (n=30) | 21.1 ± 3.3 | 19.9 ± 2.4 (n=9) | 19.8 ± 2.7 (n=7) | 18.1 ± 2.1* [#] (n=44) |
| White blood cell count | 5.6 ± 1.83 (n=1026) | 5.0 ± 1.4 (n=28) | 5.2 ± 1.5 (n=16) | 4.8 ± 1.2 (n=8) | 4.8 ± 1.3 (n=7) | 7.2 ± 2.6* [#] (n=39) |
| Mean corpuscular volume (fL) | 84.9 ± 7.7 (n=1026) | 82.6 ± 7.5 (n=28) | 83.4 ± 6.9 (n=16) | 84.5 ± 5.1 (n=8) | 84.4 ± 5.4 (n=7) | 80.2 ± 7.6* [#] (n=39) |

sTfR=soluble transferrin receptor; PTB, pulmonary tuberculosis; TB=tuberculosis; TBCC=Gambian TB Case-Contact study
Data are presented as median (IQR) or mean \pm standard deviation, % (n/n). When missing data occurred, the sample size is indicated in the table.

There were no significant differences between:

- TB-progressors in TBCC compared to TB-progressors in the TBCC subjects with available plasma
- Non-progressors in TBCC compared to non-progressors in the TBCC subjects with available plasma
- TB-progressors and non-progressors in the TBCC subjects with available plasma only

Statistical differences were assessed using Student's *t* tests for normally distributed variables and Mann-Whitney U-tests for non-normally distributed variables; Chi-Squared tests were used to compare frequencies.

* Significantly different ($P<0.05$) when active TB cases compared to non-progressors from TBCC or the TBCC subjects with available plasma.

Significantly different ($P<0.05$) when active TB cases compared to TB-progressors from TBCC or the TBCC subjects with available plasma

A dose-response relationship for concentrations of iron homeostasis biomarkers in non-progressors, early or delayed TB-progressors and active TB is shown in Figure 4.2. The lowest transferrin concentrations and highest hepcidin or ferritin concentrations were observed in active TB cases, followed by early TB-progressors, delayed TB-progressors and lastly, non-progressors. Each iron homeostasis biomarker had statistically significant associations within this pattern, but not all comparisons within the sequence were statistically significant. Earlier TB progression appears to be linked to significantly higher concentrations of hepcidin ($p=0.036$) or ferritin ($p=0.027$) compared to delayed TB-progressors among all TB-progressors. The direction and magnitude of effects were similar when including or excluding the two HIV-seropositive TB progressors, however the associations were no longer statistically significant.

While a reference range has not yet been established for hepcidin, median/mean values of plasma ferritin, sTfR and Hb were within the normal reference ranges established for iron status. Only mean transferrin concentration was below normal (27) in early and delayed TB-progressors and also in active TB cases (Figure 4.2). Lower transferrin was associated with a greater likelihood of incident TB [all TB-progressors Incidence Rate Ratio (IRR): 0.55; 95% confidence interval (CI): 0.35 to 0.89] (Table 4.2). Excluding the two HIV-infected TB-progressors from analysis did not change the magnitude or direction of effect; however, the confidence interval increased and the association was no longer statistically significant. Although not statistically significant, an IRR greater than 1.00 indicated that higher hepcidin was associated with a greater probability of progression to TB (IRR: 1.27), and an IRR below 1.00 suggested that higher BMI was associated with a lower probability (IRR: 0.75).

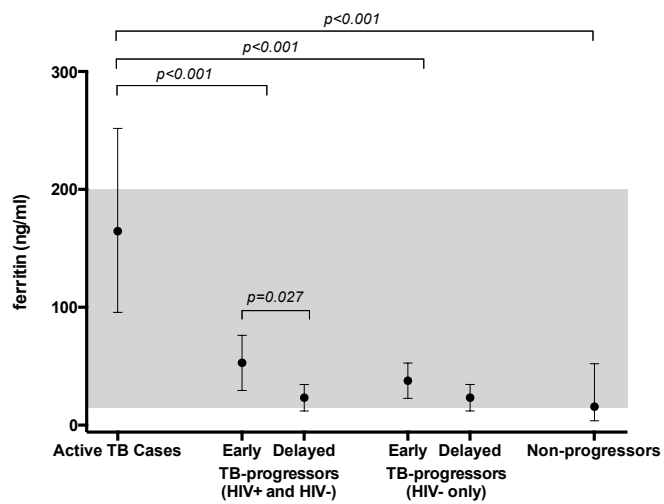
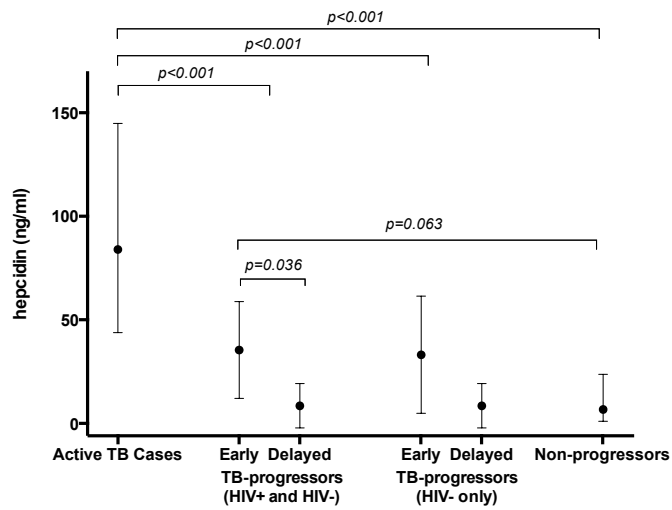
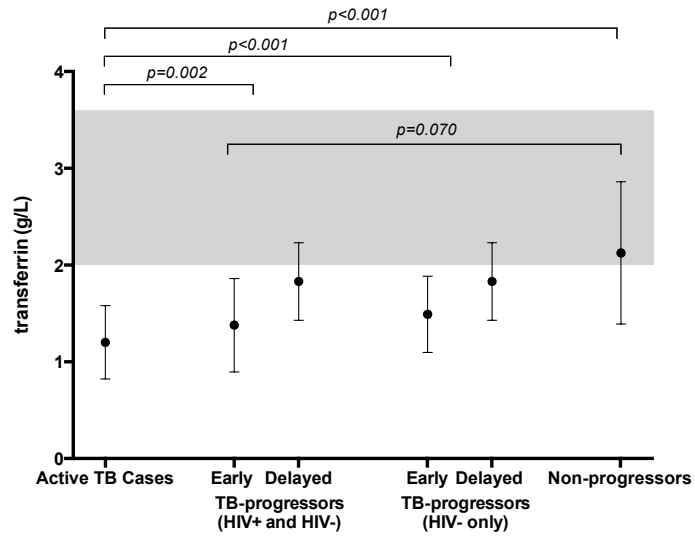
Figure 4.2 Iron homeostasis at enrollment among active tuberculosis cases prior to treatment initiation and household contacts that became early TB-progressors (3 to 24 months), delayed TB-progressors (24 to 48 months) or remained non-progressors

TB=tuberculosis; (HIV+)=HIV-infected; (HIV-)=HIV-uninfected

Early and Delayed TB-progressors (HIV+ and HIV-) includes HIV-infected TB-progressors (n=2); these HIV-infected TB-progressors were excluded in Early and Delayed TB-progressors (HIV-only).

Shaded area represents the normal reference range established for ferritin, soluble transferrin receptor, transferrin and hemoglobin (27). Parallel dotted lines at hemoglobin=12 g/L and hemoglobin=13 g/L represent the lower cutoffs for normal hemoglobin in women and men respectively (28). The hepcidin box does not have a shaded area since there is no established reference range.

P-values <0.10 are noted, and were determined using unpaired Student's *t*-tests (for comparisons between normally distributed samples) and Mann-Whitney U tests (for comparisons between non-normally distributed samples).



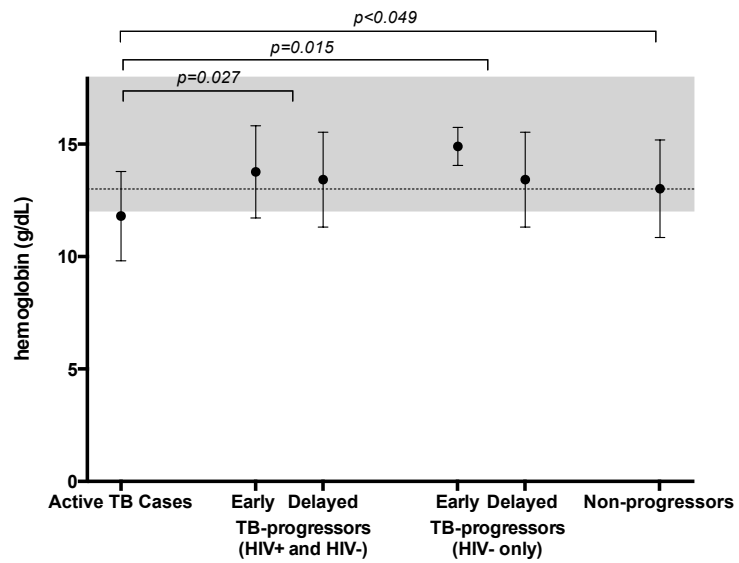
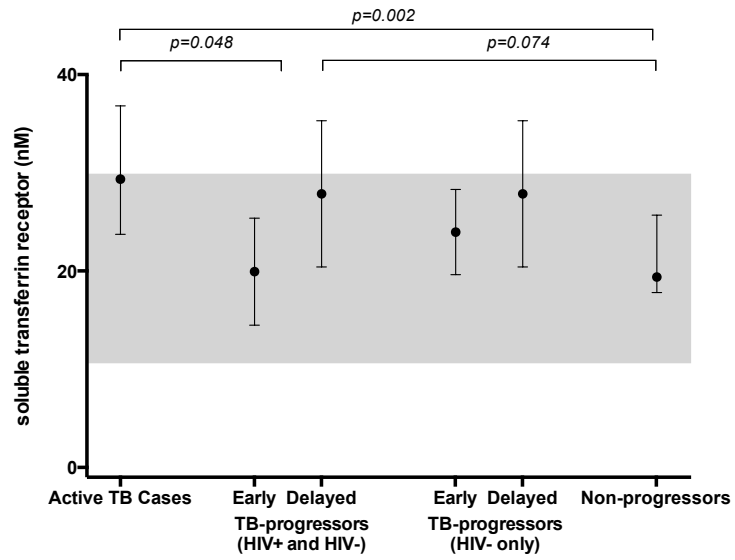


Table 4.2. Poisson regression models of the association between standardized^a iron homeostasis biomarkers, standardized body mass index^a and progression to pulmonary tuberculosis

| | All TB-progressors included | | | Only HIV-negative TB-progressors included | | |
|------------------------------|------------------------------------|----------------------------------|---------|---|----------------------------------|---------|
| | TB-progressors/ non-progressors | Incidence rate ratio (95% CI) | P-value | TB-progressors/ non-progressors | Incidence rate ratio (95% CI) | P-value |
| Transferrin | 10/17 | 0.55 (0.35 to 0.89) | 0.015 | 8/17 | 0.58 (0.31 to 1.10) | 0.096 |
| Hepcidin | 10/17 | 1.27 (0.86 to 1.87) | 0.226 | 8/17 | 1.11 (0.65 to 1.89) | 0.713 |
| Ferritin | 10/17 | 0.98 (0.63 to 1.51) | 0.913 | 8/17 | 0.78 (0.49 to 1.31) | 0.373 |
| Soluble transferrin receptor | 10/17 | 0.99 (0.65 to 1.53) | 0.982 | 8/17 | 1.09 (0.71 to 1.71) | 0.672 |
| Hemoglobin | 9/16 | 1.20 (0.68 to 2.13) | 0.526 | 7/16 | 1.37 (0.71 to 2.62) | 0.351 |
| Body mass index | 9/16 | 0.75 (0.43 to 1.31) | 0.317 | 7/16 | 0.72 (0.36 to 1.43) | 0.352 |

CI=confidence interval

^aAll biomarkers have been rescaled with mean=0; standard deviation=1.

Discussion

Findings from this study suggest that iron homeostasis is linked to TB progression in household contacts of infectious TB cases. TB progression at any time point was associated with reduced baseline concentrations of transferrin, and early TB-progressors had higher baseline concentrations of ferritin and hepcidin when compared with delayed TB-progressors. The study findings are consistent with a hypothesized functional role for iron in TB pathogenesis. Moreover, monitoring iron homeostasis biomarkers in TB contacts may be a useful indicator, in conjunction with other early-onset biomarkers, to identify individuals at greater risk for progression to TB.

These observations are largely consistent with animal and *in vitro* studies linking addition of iron to increased *Mtb* growth (16), and also with evidence indicating elevated ferritin and hepcidin along with decreased transferrin and hemoglobin, are risk factors for incident TB in a Gambian HIV-seropositive cohort (14, 15). They also add to compelling evidence for a role for iron in TB pathogenesis. Specifically, our data support a hypothesis in which increased access to iron for *Mtb* siderophores leads to improved success for the pathogen. At the crux of this hypothesis is the hypothesis that ‘too much iron’, or likely too much iron sequestered in macrophages as ferritin or bound to circulating transferrin, leads to increased TB susceptibility via improved efficiency of *Mtb* iron acquisition. Host-acquired iron is a co-factor for proteins involved in vital *Mtb* cellular processes including respiration, electron transport, DNA replication, and stress resistance. Varying levels of iron within the *Mtb*-occupied phagosome influences the activity of the *Mtb* iron-dependent regulators *furA*, *furB*, SirR and IdeR. These regulators promote and repress the transcription of certain *Mtb* genes including

the oxidative stress response protein *katG*, the iron storage proteins *bfrA* and *bfrB* and the protein machinery involved siderophore biosynthesis (reviewed in (21)). *Mtb* siderophores access multiple host iron sources including ferritin and transferrin (29), are essential for *Mtb* virulence (19) and are capable of increasing iron availability in the mycobacterial phagosome almost 20-fold (30). In an iron-limited environment the *Mtb* iron dependent regulator IdeR promotes siderophore biosynthesis in human macrophage-like THP-1 cells (31). Conversely, in the presence of sufficient iron, IdeR represses siderophore biosynthesis (in culture) and positively regulates synthesis of the iron storage protein *bfrA* (31). The latter suggests a possible effort by *Mtb* to redistribute limited resources away from iron acquisition under conditions of iron sufficiency and implies that increased efficiency of iron acquisition could lead to greater *Mtb* success. Our findings are consistent with a scenario in which increased access to ferritin (possibly due to hepcidin-mediated macrophage iron retention) and saturated transferrin for *Mtb* siderophores improves the efficiency of iron-acquisition and creates an opportunity for *Mtb* to shift resources toward growth and progression to active TB disease. If evidence from future studies lend support to this hypothesis, investigations into mechanisms that influence *Mtb* siderophore production and activity as well as host iron storage and transport, including the role of transferrin polymorphisms (32), may be warranted.

The ability to identify who is at risk for transitioning from an infected and stable state to an infected but disease progressing state is critical for both the clinical management of the individual and for the prevention of TB transmission at the population level. While the current study aimed to make this identification from the iron homeostasis biomarker perspective, previous efforts in The Gambia and elsewhere have focused on the demographic, clinical,

microbiological, immunological and genetic perspectives. Known household exposure to TB, smoking and ethnicity increased the likelihood of active TB disease in a Gambian case-control study (33). In Uganda, a case-contact study identified both young children and HIV-infected individuals as being particularly vulnerable to TB progression (34). From the microbiological perspective, analysis of *Mycobacterial* lineage in The Gambia suggested that pathogen differences influence progression to active TB (35). Also, in The Gambia, previous analysis of TB-progressors and non-progressors from the TBCC platform indicated that progressors tended to have increased levels of the cytokine IL-18, decreased expression of the anti-apoptotic gene, Bcl2 and increased expression of the gene encoding CCR7(36). Combined with the results from the current study, these previous findings suggest that strategic monitoring of individuals prior to TB diagnosis may be useful in identifying who is at risk for progression to TB.

Importantly, while the findings from the current study indicate that monitoring iron status, and in particular, transferrin, ferritin and hepcidin, may be useful in identifying who is at risk of TB progression, the described pattern of biomarkers would not be identified during routine screening for iron status. Only Hb is routinely measured in a clinical setting, and Hb, as well as sTfR and ferritin concentrations were within normal ranges (27). A reference range for hepcidin has not been established. Further studies are warranted to determine if the pattern of iron biomarker concentrations identify TB susceptibility, and if so, determine TB-specific versus anemia or iron-status thresholds or cutoffs.

A major strength of this study is its unique design, which presented a rare opportunity to investigate biomarkers as risk factors for susceptibility to TB rather than as factors associated with TB diagnosis or prognosis. However, the findings must also be considered in the context of

the study limitations. The number of TBCC participants with available plasma samples was small and restricted to a mixture of HIV-infected and HIV-uninfected individuals. The small sample size also precluded subgroup analyses according to HIV status, and full consideration of potential confounding/modifying variables like age, smoking status, BMI and contact proximity to the index PTB case. Further studies will be needed to further clarify and confirm the key study observations.

Conclusions

The probability of TB progression following infection appears to be linked to a distinct pattern of iron homeostasis biomarkers. This pattern is representative of perturbations in iron homeostasis that are consistent with a plausible biological mechanism leading to TB progression, but would not be identifiable in routine assessment of iron status that typically includes Hb. Targeted monitoring of iron homeostasis biomarkers in TB contacts may help to identify those at greater risk for progression to TB, thereby reducing delays in TB diagnosis and/or treatment to improve TB-related health outcomes and decrease TB transmission.

Further studies are needed to validate the mechanistic links between these biomarkers and HIV-associated and non-HIV-associated TB pathogenesis, and to determine if iron homeostasis biomarkers do indeed have clinical utility.

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CHAPTER 5

Complex anemia in tuberculosis: the need to consider causes and timing while designing interventions*

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Abstract

Background: Anemia is common in tuberculosis and multiple etiologies necessitate targeted interventions. The proportion of iron-responsive anemia due to iron deficiency compared to iron-unresponsive anemia due to impaired iron absorption/redistribution from tuberculosis-associated immune activation or inflammation is unknown. This impedes selection of safe and effective treatment and appropriate intervention timing.

Methods: Baseline hemoglobin, ferritin, hepcidin, soluble transferrin receptor (sTfR) and transferrin were measured in 45 confirmed pulmonary tuberculosis cases, 47 tuberculin skin test (TST)-positive controls and 39 TST-negative controls in The Gambia. TB cases were additionally followed two and six months after tuberculosis treatment initiation. Mutually exclusive anemia categories based on iron biomarker concentrations were iron-deficiency anemia (IDA), anemia of inflammation (AI) and multifactorial anemia (IDA+AI).

Results: Anemia was more frequent in tuberculosis cases (67%) compared to TST-positive (36%) or TST-negative (21%) controls. AI was the predominant anemia at tuberculosis diagnosis, declining from 36% to 8% after six months of treatment; however, a corresponding reduction was not evident for anemia with iron-responsive components (IDA, IDA+AI). Iron biomarkers discriminated between active tuberculosis and TST-positive or TST-negative controls, as well as between active untreated and treated tuberculosis. This was most noticeable for hepcidin, which decreased from a median of 84.0 ng/ml at diagnosis to 9.7 ng/ml after two months ($p<0.001$).

Conclusions: Tuberculosis chemotherapy is associated with significant reductions in AI but IDA and IDA+AI remain unresolved. Iron-based interventions are needed for IDA and IDA+AI, and

monitoring iron biomarkers reveals a window for intervention opening as early as two months into tuberculosis treatment.

Introduction

Anemia affects a quarter of the world's population (1), and sub-Saharan Africa is disproportionately burdened (2). Like all clinical conditions, resolution requires effective interventions to appropriately address etiologies. Anemia results from a number of causes acting alone or in combination (2), however, untangling the causative factors in order to effectively target anemia interventions is rarely conducted in resource-restricted regions. These challenges contribute to the sustained high level of global anemia.

Iron-based interventions are effective in treating iron-deficiency anemia (IDA) (3-5), but ineffective (6), unnecessary (7) and potentially unsafe (8, 9) when anemia of inflammation (AI) due to infection is present. Providing sufficient dietary iron to meet iron requirements will resolve an iron supply problem (i.e. iron-responsive anemia), but will not resolve an iron delivery problem where absorbed dietary iron is blocked from release from intestinal enterocytes and body iron is shuttled to and retained in the macrophage. This iron delivery problem is a hallmark of AI (i.e. iron non-responsive anemia) that requires resolution of the underlying infectious or inflammatory pathology (10). Regions with high dual burdens of infectious diseases and malnutrition present a particular challenge in identifying when and how to treat existing anemia given that AI and IDA require such different interventions. This challenge is especially salient in sub-Saharan Africa, where a mixture of multifactorial IDA+AI anemia may constitute more than 50% of anemia (2).

Anemia is a common comorbidity at tuberculosis (TB) diagnosis, with prevalence estimates ranging from 32% to 86% (11-16). While not specifically investigated, a large proportion of tuberculosis anemia is likely due to AI. This is supported by data from two Asian

studies where the prevalence of anemia at TB diagnosis fell by 67 and 88% upon completion of TB chemotherapy without additional iron-based anemia interventions (14, 15). While this is an important reduction in overall anemia, there were still a considerable number of participants with unresolved anemia by the end of TB treatment (19.9 and 7.6%, respectively), suggesting IDA or multifactorial IDA+AI was also present at TB diagnosis.

Given the consequences of unresolved anemia in general (17-20), evidence pointing to a relationship between anemia, iron redistribution and TB susceptibility (21, 22) and reports linking anemia to poor clinical prognoses and mortality after TB diagnosis (16, 23, 24), further understanding of anemia management in TB is needed. Thus, this study was designed to: 1) characterize the relative contributions of anemia with iron-responsive components (IDA, IDA + AI) and iron non-responsive components (AI) to inform selection of the most appropriate treatment approach; 2) evaluate the evolution of biomarkers of iron status during TB therapy to identify the best timing of targeted anemia interventions in TB; and 3) investigate risk factors for TB anemia in The Gambia.

Methods

Adult participants were recruited following written informed consent as part of the ongoing TB Case-Contact study at the Medical Research Council Unit in The Gambia (25). 45 culture-confirmed Pulmonary TB cases and 86 *Mycobacterium tuberculosis* Complex (MTBC)-exposed contacts were recruited between October 2010 and May 2012, and cases were followed until completion of TB treatment. Tuberculin skin tests (TST) were performed to determine the infection status of all contacts using two tuberculin units (TU) of purified protein derivative (PPD). An induration >10mm was considered TST-positive (n=47) and ≤10mm TST-

negative (n=39) due to Bacille-Calmette Guerin (BCG) vaccination and high prevalence of environmental mycobacteria in this setting. Sputum samples were stained with auramine and the Ziehl-Nielsen method. Sputum was decontaminated using N-acetyl cysteine (NALC)-NaOH before culturing in Bactec vials (Becton Dickinson, USA) and on paired Lowenstein-Jensen slopes. Positive cultures were confirmed by Ziehl-Nielsen smear.

All TB cases underwent a clinical assessment at recruitment (defined as TB diagnosis) and at two and six months following TB treatment initiation. The clinical assessment included determination of disease severity by chest x-ray, comorbidity assessment and TB-specific clinical questions such as the presence of side pain, cough duration and weight loss. Malaria screening was conducted using a rapid diagnostic test, and those with malaria were excluded from participation. Blood samples were obtained from TB cases at all three time points and at baseline only for TST-positive and TST-negative groups with storage at -70°C until analysis. At the time these data were collected, TB treatment success rate in The Gambia was >85% (26) and national TB treatment guidelines included an intensive phase of isoniazid, rifampicin, ethambutol, and pyrazinamide for two months followed by isoniazid and rifampicin for four months (27).

Demographic, Anthropometric and Clinical Characteristics

Data was collected on participant age, gender, body mass index (BMI), mid-upper arm circumference (MUAC), HIV-status, hemoglobin (Hb), white blood cell count (WBC) and mean corpuscular volume (MCV).

Iron Status Biomarkers and Anemia Classification

Plasma or serum ferritin (Immuno-biological Laboratories, Germany), soluble transferrin receptor (sTfR) (R&D Systems, UK) and transferrin (Tf) (Cygnus Technologies, USA) were measured by ELISA. Hepcidin was measured using a competitive enzyme immunoassay (Bachem, USA). All biomarker assays were optimized for use with plasma and serum and a sensitivity analysis confirmed specimen type did not affect interpretation. Test samples, standards, and controls were assayed in duplicate and concentrations interpolated from 4-parameter logistic standard curves (log/log curves for sTfR). All standard curves were generated using SoftmaxPro 6 (Molecular Devices, USA). Samples with an intra-assay coefficient of variation >15%, were re-assayed. Lower limits of detection (LoDs) for all assays, with the exception of hepcidin, were defined by the manufacturer. For hepcidin, the LoD (0.2 ng/ml) was interpolated at three standard deviations from the all plate mean (e.g. wells that contained diluent in lieu of hepcidin standard or sample). Anemia was defined according to World Health Organization criteria for males Hb <13 g/dL and females Hb <12 g/dL (28), while iron deficiency was defined as MCV <80 fL (29). Anemia classification was based on mutually exclusive criteria as follows: 1) AI was defined as anemia plus sTfR/log(ferritin) ratio <14 (30); 2) IDA was defined as anemia plus iron deficiency without AI; and 3) IDA+AI was defined as IDA plus AI.

Statistical Analysis

Statistical analyses were performed using STATA 13.0 (College Station, USA) and Prism 6.0 (La Jolla, USA). Median values of each biomarker were compared using the Wilcoxon matched pairs signed rank test for non-parametric comparisons at different time points (for cases only) and the Mann-Whitney *U*-test for comparisons of non-matched subjects (cases

compared to TST-positive and TST-negative contacts). Paired and unpaired Student's *t* tests were similarly used for normally distributed variables at different time points and for non-matched subjects, respectively. Chi-square and Fisher's Exact tests were used to compare categorical variables. Logistic regression was used to model the relationships between disease status, iron parameters and the binary variables of anemia, IDA, AI and IDA+AI in a cross-section of the data that included TB cases, TST-positive and TST-negative data at baseline. Random-effects multi-level logistic modeling to account for repeated observations within individuals was used to examine relationships between binary variables of anemia, IDA, AI, IDA+AI and iron biomarkers with disease-related variables including treatment duration and disease radiographic severity among the TB cases during TB treatment. Multiple imputed values for anemia, IDA, AI and IDA+AI were included in the multi-level logistic regression model. Missing data followed a monotone-missing pattern indicating the absence of a value for one variable implied missing values for another variable. Imputation was performed as a sequence of univariate (conditional) imputation tasks, covariates included in the imputation models were: age, gender, BMI, MUAC, hepcidin, sTfR, ferritin and Tf. Participants with confirmed HIV seropositivity were antiretroviral naive and their disease stage or absolute CD4 cell count was unavailable. HIV status was unconfirmed for 32 participants, and to determine the effect on these data, a sensitivity analysis was conducted. Two extreme considerations were examined where all individuals with unknown HIV status were coded as "HIV positive" and then all as "HIV negative". Differences in interpretation including direction of effect, magnitude or statistical significance were not evident, and additionally, including only those individuals with confirmed HIV status (e.g. confirmed HIV-positive and confirmed HIV-negative) in models did not indicate

HIV infection was a significant confounder in these data. Based on these analyses, HIV status was not included as an independent variable in final regression models.

Results

Characteristics of TB cases and contacts are summarized in Table 5.1. As expected, TB cases were experiencing significantly poorer health at TB diagnosis based on nutritional (lower BMI, MUAC), hematological (lower Hb and higher WBC) and iron status (higher ferritin, hepcidin, sTfR and lower Tf) parameters compared to TST-positive or TST-negative contacts (Table 5.1).

Classification of anemia may inform anemia treatment in TB

All-cause anemia was a common problem diagnosed in many study participants (41%, $n=51/125$). AI accounted for 45% ($n=23/51$) of anemia amongst all participants (combined TB, TST-negative, TST-positive) and it was the most common anemia classification at TB diagnosis with more than 1/3 of TB cases experiencing AI (36%, $n=14/39$) (Table 5.1). While TB cases were significantly more likely to have AI at diagnosis than TST-negative/TST-positive combined (36% vs. 21%, respectively, $p=0.002$), the proportions of participants with IDA (18% vs. 12%, respectively, $p=0.40$) and IDA+AI (13% vs. 7%, respectively, $p=0.32$) were not significantly different (Table 5.1).

Risk factors associated with all-cause anemia and cause-specific anemia including IDA, AI and IDA+AI in TB cases at diagnosis and contacts (TST+ and TST-) are summarized in Figure 5.1. AI, but not IDA or IDA+AI, was significantly associated with TB in a logistic regression model, indicating that AI was an important contributor to the observed association between TB and all-cause anemia at diagnosis.

Longitudinal assessment of TB anemia summarized in Table 5.2 indicated that TB

treatment for six months was associated with a significantly lower likelihood of all-cause anemia (adjusted OR=0.35, 95% CI=0.14 to 0.86), as well as AI (adjusted OR=0.19, 95% CI=0.06 to 0.67) compared to TB diagnosis. In contrast, TB treatment was not associated with anemia resolution if baseline anemia had an iron-responsive component classified as IDA or IDA+AI at TB diagnosis (Table 5.2).

Iron status biomarkers change significantly during TB treatment

At TB diagnosis, concentrations of iron biomarkers in TB cases differed significantly from TST-positive and TST-negative contacts (Table 5.1, Figure 5.2) and changed considerably throughout TB treatment (Figure 5.3). Concentrations of ferritin, hepcidin and sTfR were found to be significantly higher in active but untreated TB cases at TB diagnosis compared to TST-negative and TST-positive contacts combined, while concentrations of Tf and hemoglobin were significantly lower (Figure 5.2). Ferritin, hepcidin and sTfR were significantly lower following two months of TB treatment (Figure 5.3), and no longer statistically different from TST-negative contacts after six months of TB treatment (Table 5.1). Tf and hemoglobin concentrations increased during treatment to levels that were not statistically different from TST-negative contacts (Table 5.1 and Figure 5.3). A similar pattern was evident in a random-effects multi-level logistic regression model adjusted for age and sex. The odds of elevated ferritin (OR=0.02, 95% CI=0.003 to 0.17), elevated hepcidin (OR=0.03, 95% CI= 0.006 to 0.18), elevated sTfR (OR=0.15, 95% CI=0.03 to 0.71) and low Tf (OR=0.23, 95% CI=0.07 to 0.71) were significantly lower after two months of treatment vs. diagnosis, and after 6-months vs. diagnosis (elevated ferritin: OR=0.019, 95% CI=0.002 to 0.16; elevated hepcidin: 0.075, 0.02 to 0.31; elevated sTfR: 0.11, 0.02 to 0.54; low Tf: 0.11, 0.03 to 0.39).

Table 5.1. Participant characteristics amongst *Mycobacterium tuberculosis* Complex-infected (TST-positive) and uninfected (TST-negative) contacts at study recruitment and amongst TB cases at TB diagnosis and following two and six months of TB treatment

| | TST-negative n=39 | TST-positive n=47 | TB at diagnosis, n=45 | TB at 2-months, n=43 | TB at 6-months, n=44 |
|---|----------------------------|----------------------------|-------------------------|----------------------|----------------------|
| Age, years | 23 (20 to 30) | 28 (24 to 47) | 29 (23 to 38) | | |
| male, % | 46% (18/39) | 49% (23/47) | 62% (28/45) | | |
| HIV-negative, % | 80% (31/39) | 51% (24/47) | 89% (40/45) | | |
| HIV-positive, % | 2% (1/39) | 2% (1/47) | 4% (2/45) | | |
| HIV-unknown, % | 18% (7/39) | 47% (22/47) | 7% (3/45) | | |
| Nutritional status indicators | | | | | |
| Body mass index (BMI) (kg/m ²)* | 20.6 (18.9 to 23.9) (n=37) | 22.2 (20.3 to 24.5) (n=46) | 18.1 ± 2.1 (n=44) | 19.2 ± 2.2 (n=39) | 20.1 ± 2.0 (n=19) |
| Mid-upper arm circumference (MUAC) (cm)* | 26.0 (24.9 to 28.3) (n=38) | 26.5 (25.0 to 28.0) (n=46) | 23.1 ± 2.2 (n=44) | 24.5 ± 2.5 (n=43) | 25.0 ± 2.2 (n=24) |
| Hematological indicators | | | | | |
| Hemoglobin (Hb) (g/dL)* | 13.3 (12.5 to 14.8) | 13.2 (11.5 to 14.4) | 11.8 ± 2.0 (n=39) | 12.6 ± 2.2 (n=7) | 13.4 ± 1.7 (n=41) |
| White blood cell count (WBC)* | 5.5 ± 1.2 | 5.6 (4.7 to 6.6) | 6.9 (5.3 to 8.6) (n=39) | 4.8 ± 0.6 (n=7) | 5.2 ± 1.5 (n=41) |
| Mean corpuscular volume (MCV) (fL) | 83.8 (81.3 to 87.2) | 80.9 ± 8.1 | 80.2 ± 7.6 (n=39) | 81.7 ± 6.4 (n=7) | 82.3 ± 8.2 (n=41) |
| Iron indicators | | | | | |
| Ferritin (ng/ml)* | 21.9 (11.4 to 39.0) | 36.4 (8.9 to 62.1) | 164.5 (95.7to 252) | 24.0 (9.1 to 76.3) | 29.2 (11.6 to 89.2) |
| Hepcidin(ng/ml)* | 22.9 (5.9 to 41.7) | 24.1 (2.4 to 45.4) | 84.0 (43.8 to 145) | 9.7 (3.9 to 53.0) | 20.4 (2.5 to 65.1) |
| Transferrin (g/L)* | 1.8 (1.5 to 2.3) | 1.9 (1.5 to 2.1) | 1.2 ± 0.38 | 1.5 (1.1 to 2.0) | 11.9 ± 0.63 |
| sTfR (nmol/L)* | 18.5 (15.6 to 22.0) | 23.2 (16.7 to 27.2) | 29.36 (23.7 to 37) | 24.1 ± 11.6 | 24.7 (19.1 to 31.2) |

| Iron deficiency and anemia classification | | | | | |
|---|------------|-------------|-------------|-----------|-------------|
| Iron Deficiency [#] | 23% (9/39) | 45% (21/47) | 51% (20/39) | 43% (3/7) | 34% (14/41) |
| Anemia [#] | 21% (8/39) | 36% (17/47) | 67% (26/39) | 57% (4/7) | 41% (17/41) |
| AI [#] | 8% (3/39) | 13% (6/47) | 36% (14/39) | 29% (2/7) | 8% (4/40) |
| IDA | 5% (2/39) | 17% (8/47) | 18% (7/39) | 14% (1/7) | 18% (7/40) |
| IDA+AI | 8% (3/39) | 6% (3/47) | 13% (5/39) | 14% (1/7) | 15% (6/40) |

Values are reported as median (IQR), mean \pm SD, or % (n)

IQR= interquartile range; n= sample size; MUAC= mid-upper arm circumference; Hb= hemoglobin; WBC= white blood cells; MCV= mean corpuscular volume; BMI=Body Mass Index; sTfR= soluble transferrin receptor; AI= Anemia of Inflammation; IDA= iron deficiency anemia

Anemia= male Hb <13 g/dL, female Hb <12 g/dL

Iron deficiency= MCV <80 fL

AI= individuals who are anemic plus have an sTfR/log(ferritin) index < 14

IDA= individuals who are anemic plus iron deficient, but were not classified as AI

IDA+AI= individuals who are IDA plus have an sTfR/log(ferritin) index \geq 14, but not classified as AI or IDA only

sTfR-log/ferritin index is calculated as sTfR (nmol/L)/log₁₀(ferritin (ng/ml))

*Values for TB cases at diagnosis were significantly different compared with non-TB cases (TST-positive and TST-negative contacts combined) (Mann-Whitney test; $P < 0.05$)

[#]Conditions were significantly more frequent in TB cases (at diagnosis) vs. non-TB cases (TST-positive and TST-negative contacts combined)(Fisher's exact test; $P < 0.05$)

Values of nutritional status indicators, hematological indicators and iron indicators for TB cases after six months of treatment were *not* statistically different from those observed in TST-negative contacts (Mann-Whitney test; $P < 0.05$)

Table 5.2. Predictors of anemia etiologies using multiply imputed values for anemia, IDA, AI, and IDA+AI amongst TB cases throughout TB treatment

| | Anemia | IDA | AI | IDA+AI |
|-------------------------------------|------------------------|------------------------|------------------------|------------------------|
| <i>Duration of treatment</i> | | | | |
| TB diagnosis | Reference | | | |
| 2-Months | 0.84 (0.33 to 2.1) | 1.27 (0.36 to 4.52) | 0.84 (0.33 to 2.18) | 2.07 (0.76 to 5.67) |
| 6-Months | 0.35 (0.14 to 0.86) | 0.82 (0.23 to 2.93) | 0.19 (0.06 to 0.67) | 0.85 (0.28 to 2.61) |

Mixed-effects logistic regression was utilized to model the binary outcomes of anemia, IDA, AI, and IDA+AI. Models were adjusted for age, gender and TB treatment duration.

Forty-five TB cases having samples from three time points were used in this analysis providing a total of 135 possible data points. Due to missing data and model convergence, the total number of data points utilized in this model ranged from 125 to 133.

Anemia= male Hb <13 g/dL, female Hb <12 g/dL (28)

Iron deficiency= MCV <80 fL (29)

AI= individuals who are anemic plus have an sTfR/log(ferritin) index < 14 (30)

IDA= individuals who are anemic plus iron deficient, but were not classified as AI

IDA+AI= individuals who are IDA plus have an sTfR/log(ferritin) index ≥ 14 , but not classified as AI or IDA only

sTfR-log/ferritin index is calculated as sTfR (nmol/L)/log₁₀(ferritin (ng/ml))

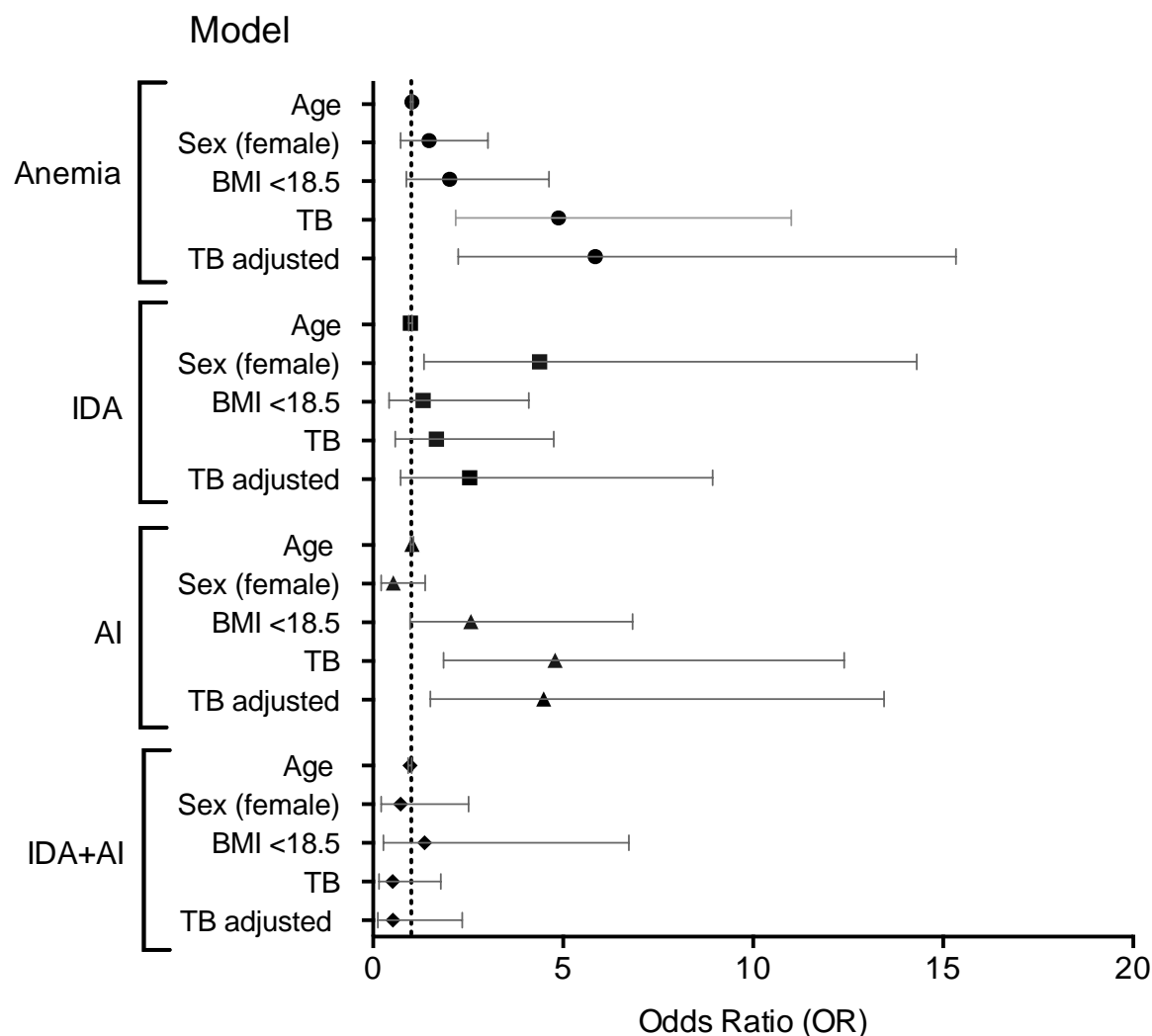


Figure 5.1. Risk factors for all-cause anemia and cause-specific anemia in 125 participants with active TB cases at TB diagnosis, *Mycobacterium tuberculosis* Complex infected (TST-positive) and uninfected (TST-negative) contacts at study recruitment

BMI=Body Mass Index; sTfR= soluble transferrin receptor; AI= Anemia of Inflammation; IDA= iron deficiency anemia

Univariate logistic regression was utilized to model the relationship between anemia (all forms) (n=51/125), IDA (n=19/125), AI (n=28/125) and IDA+AI (n=11/125) with age, sex, BMI (≥ 18.5 vs < 18.5) and TB (TB vs no active TB). In the “TB adjusted” regression model, the TB variable was adjusted for age, gender and BMI.

Anemia= male Hb < 13 g/dL, female Hb < 12 g/dL (28)

Iron deficiency= MCV < 80 fL (29)

AI= individuals who are anemic plus have an sTfR/log(ferritin) index < 14 (30)

IDA= individuals who are anemic plus iron deficient, but were not classified as AI

IDA+AI= individuals who are IDA plus have an sTfR/log(ferritin) index ≥ 14 , but not classified as AI or IDA only

sTfR-log/ferritin index is calculated as sTfR (nmol/L)/log₁₀(ferritin (ng/ml))

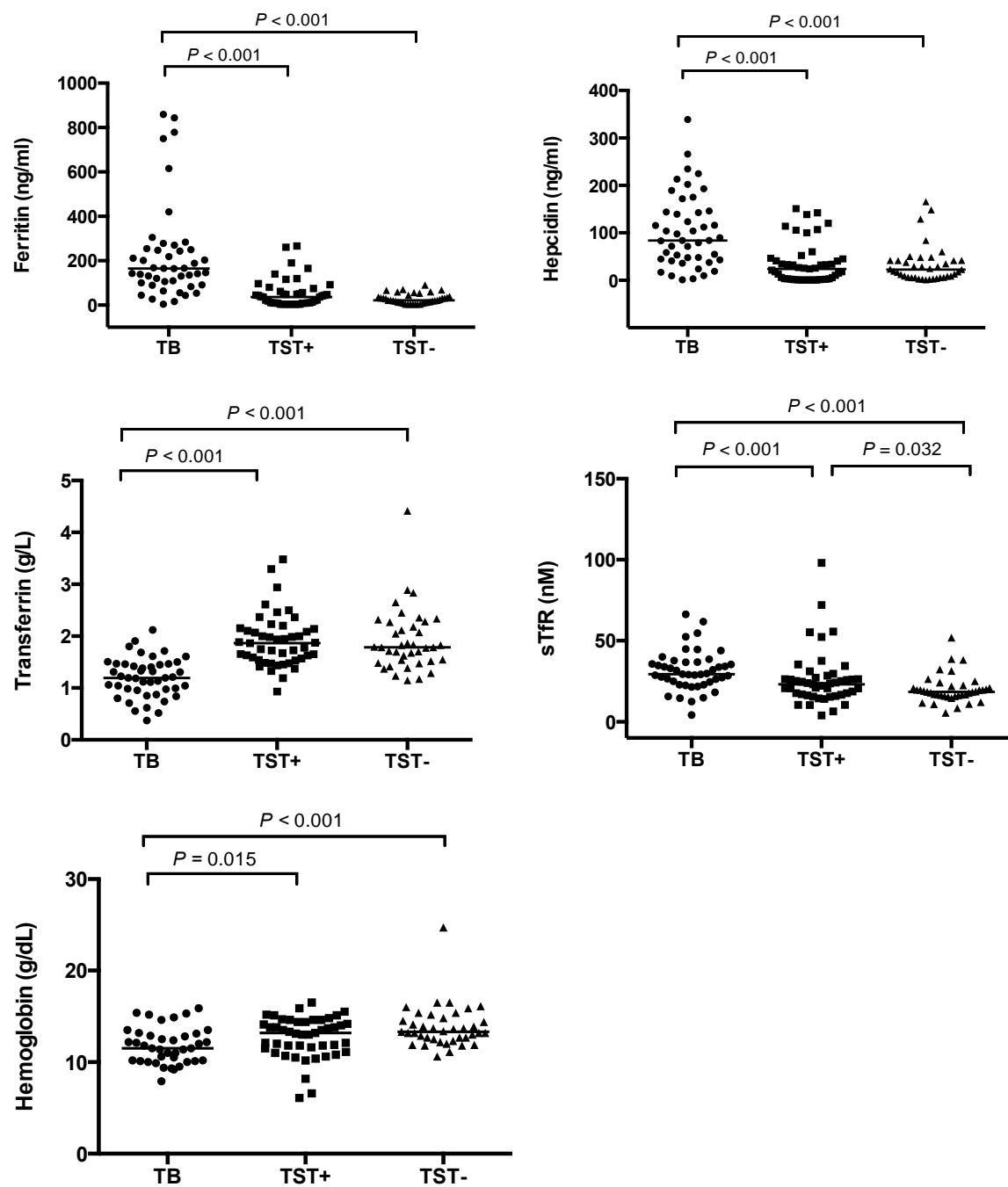


Figure 5.2. Iron status biomarkers in active TB cases at TB diagnosis, *Mycobacterium tuberculosis* Complex infected (TST-positive) and uninfected (TST-negative) contacts. Mann-Whitney test.

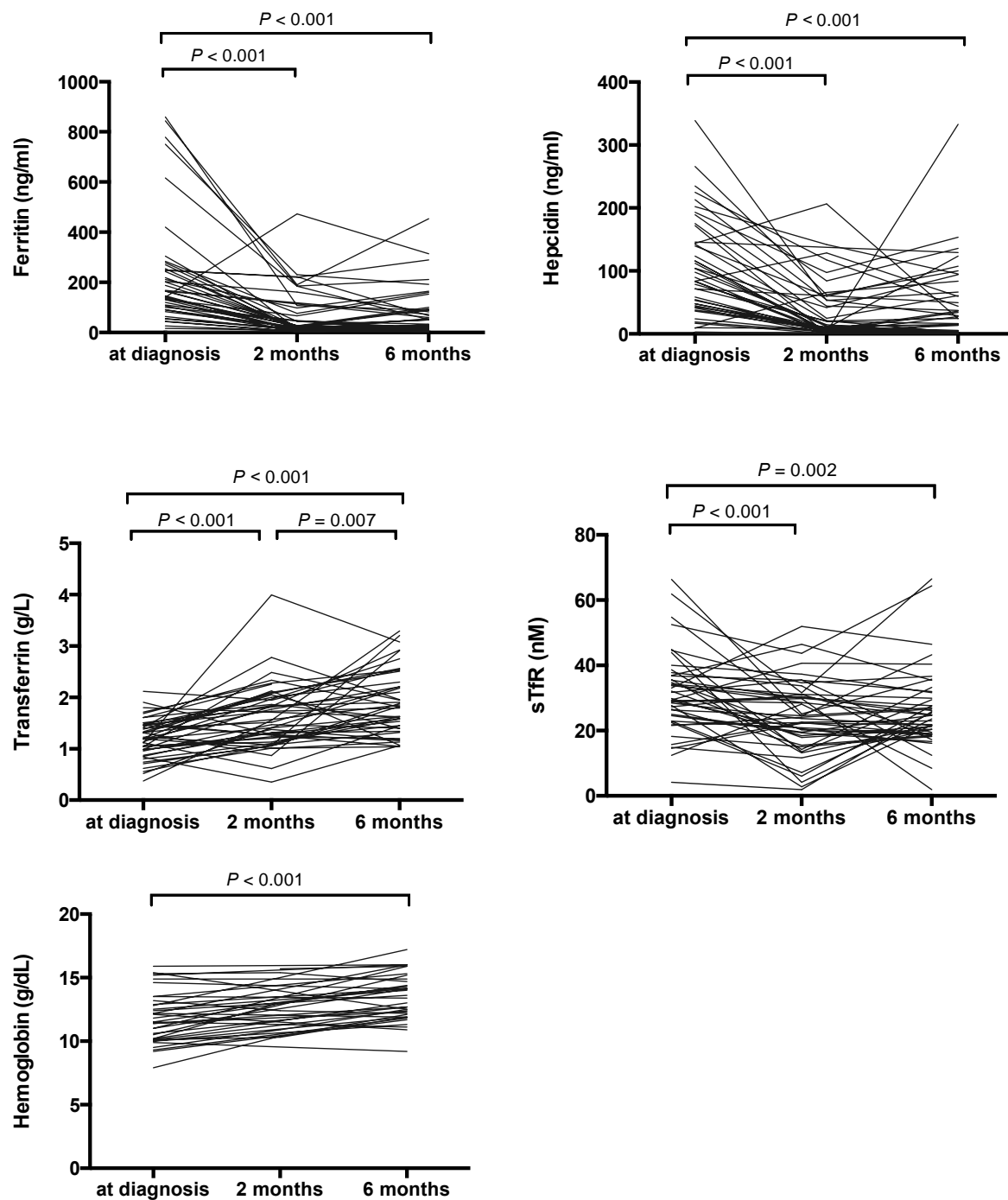


Figure 5.3. Longitudinal changes in ferritin, hepcidin, soluble transferrin receptor (sTfR), transferrin and hemoglobin concentrations among TB cases during treatment. Wilcoxon matched-pairs signed rank test.

Discussion

All-cause anemia was a common comorbidity in TB, with the majority of individuals classified as AI. While AI was largely resolved after completion of TB therapy without iron-specific interventions, this approach alone was insufficient to resolve all forms of anemia. Anemia that had a sole or combination iron-responsive component (e.g. IDA, or IDA+AI, respectively) made up almost half of all-cause anemia at TB diagnosis and remained mostly unresolved by the end of TB treatment. Resolution of iron-responsive anemia calls for iron-focused interventions, and in the context of TB and malnutrition, this study demonstrates for the first time that the window of opportunity to intervene with iron appears to open as early as two months after TB treatment initiation.

Others have also reported that anemia is a common comorbidity at TB diagnosis (11-16) and that it is partially resolved with TB treatment (14, 15), but the evidence presented here goes further suggesting AI is the predominant anemia classification at TB diagnosis. These data, while novel, are consistent with what is known about the biology of anemia. AI is a consequence of inflammation and is most often the result of inflammation-related pathologies such as short erythrocyte life-span, poor erythrocyte iron incorporation and decreased sensitivity to or supply of erythropoietin (17). IDA and IDA+AI, on the other hand, occur as a result of negative iron balance alone or in combination with inflammation, respectively. Treating TB enables recovery from AI via reduced inflammation and also likely leads to improved dietary intake. While the latter may independently contribute to normalizing iron status through improved iron balance, evidence suggests that this contribution is insufficient to resolve iron-responsive anemia.

Treating iron-responsive anemia in the presence of inflammation and infection is complicated. From a safety perspective, changes in iron metabolism under inflammatory conditions may create a favorable environment for pathogen iron acquisition and growth. The combination of iron supplementation and inflammation-mediated low Tf concentration could lead to an increase in non-transferrin bound or 'free' iron. This unbound iron has been implicated in previous reports detailing poor health outcomes following iron supplementation in settings where infection is common (31). Moreover, inflammation leads to elevated levels of the iron regulatory peptide, hepcidin (reviewed in (32)), which controls enterocyte and macrophage iron efflux. Elevated hepcidin has been shown to forecast non-responsiveness to oral iron therapy (33), suggesting that irrespective of the amount of iron being delivered, iron balance will not be restored when inflammation levels remain high. The key to resolving iron-responsive anemia in this context appears to be to first control inflammation, and then consider iron interventions at time when hepcidin levels are reduced.

By monitoring concentrations of hepcidin and other iron biomarkers throughout TB treatment, optimal timing for a possible iron intervention may be identified. In this study, high concentrations of hepcidin at TB diagnosis indicated that an iron intervention would likely be ineffective. However, the dramatic drop in hepcidin concentrations that we observed after just two months of TB treatment suggests that an opportunity to effectively and safely intervene with iron and treat iron-responsive anemia opens up prior to the completion of TB treatment. Further studies are needed to validate this strategy, determine which markers are the best to pinpoint this intervention opportunity, and to identify parameters that constitute safe and effective iron supplementation at an individual level.

An important, but elusive goal in TB treatment and control is the identification of individuals at risk to transition from MTBC-infected and stable latent TB infection (LTBI) to active TB disease. Based on the iron biomarker patterns observed in this study, which differentiated between active untreated TB cases and TST-positive contacts, iron biomarkers appear to have potential to contribute to the development of risk profiles for the transition from LTBI to TB. However, in order to properly evaluate iron-associated proteins as disease transition biomarkers a TB case-contact study with serial biological archives from TB contacts that progress to active TB is needed. Alternatively, an existing cohort set in a TB endemic region with serial biological samples and well-characterized TB-related participant characteristics may already exist. Such studies would build on existing evidence indicating that higher concentrations of ferritin (21) and hepcidin (22, 34) as well as lower concentrations of Tf (21) and hemoglobin (21) are risk factors for incident TB with HIV co-infection.

Key strengths of this study include the analysis of multiple iron biomarkers to enable specific classification of anemia types in TB contacts compared to TB cases, and in TB cases throughout treatment. These data contributes to a better understanding of appropriate treatment options and timing in settings where complex anemia from infection and nutrition-related etiologies coexist. Limitations include the lack of consensus definitions for anemia classification that restrict comparability with studies using different anemia definitions. While the sensitivity analyses suggests HIV infection was not a significant factor in this dataset, the role of HIV infection, including stage of disease or immunosuppression and HIV treatment, needs to be specifically confirmed in future research. In addition, it will be important to confirm the clinical relevance of these findings across different subgroups including hereditary

iron disorders that are uncommon in The Gambia (35), and in smokers and pre- and post-menopausal women with potentially differential iron metabolism and TB outcomes.

Conclusion

Anemia comorbidity is common in TB and predominantly due to AI at TB diagnosis. While TB treatment was associated with resolution of AI, TB treatment alone did not eliminate all AI, and the prevalence of IDA and IDA+AI was essentially unchanged following TB treatment. This underscores the importance of utilizing iron biomarkers to classify anemia type and then matching anemia interventions to causes. Changes in iron biomarker concentrations during TB treatment may inform timing of iron interventions. Importantly, hepcidin concentrations decreased dramatically after two months of treatment, revealing a potential window of opportunity for iron-based interventions aimed at resolving iron-responsive anemia. In light of this evidence, strategies to identify and treat anemia in TB should be re-evaluated to include classification of anemia and specific timing considerations that can be used to guide effective anemia intervention.

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CHAPTER 6

Conclusions and Recommendations

Contribution to the Literature

In light of evidence supporting roles for iron in HIV, HIV/TB co-infection, TB and anemia, this research aimed to characterize iron homeostasis in these contexts in order to provide insight into pathogenesis and to explore their potential clinical utility of iron homeostasis biomarkers. This was accomplished via the evaluation of iron homeostasis biomarker concentrations at HIV diagnosis, upon contact with a known infectious TB case, and throughout TB treatment.

While the relationship between hemoglobin and HIV is well characterized, few studies have expanded their inquiries to include other iron homeostasis biomarkers and even fewer have explored those biomarkers in relation to incident TB, mortality, TB susceptibility and TB-associated anemia.

This research includes one of just two studies that has examined the relationship between the iron homeostasis biomarker, hepcidin, and incident TB in HIV and it also includes the first and only study to describe the relationship between hepcidin and mortality in an HIV-seropositive cohort. The use of principal components analysis in the latter study added an extra dimension and enabled a more comprehensive understanding of the mechanisms involved. The investigation into iron homeostasis biomarkers in contacts of infectious TB cases is also novel. Additionally, this research is one of just a few studies to characterize iron homeostasis biomarkers throughout TB treatment and the first and only study to do so in such a way that informs the timing of potential anemia interventions.

Conclusions

Chapter 2

1. The highest quartile of hepcidin concentrations at HIV diagnosis accounted for >40% of all incident TB cases.
2. Comparing the upper hepcidin quartile to the lower three quartiles combined revealed a two-fold increase in the incidence of TB [unadjusted incident rate ratio (IRR); 95% CI=2.05; 1.01 to 4.16]
3. Hepcidin's ability to predict incident TB was equal to or better than covariates representing greater immunosuppression, inflammation and lower BMI.
4. Hepcidin may modulate the host environment to favor *Mycobacterium tuberculosis* and/or be a viable proxy biomarker for subclinical TB.

Chapter 3

1. Hepcidin at HIV diagnosis was positively correlated with ferritin, inflammation and immunosuppression and inversely correlated with hemoglobin and transferrin.
2. There was a dose response relationship between greater hepcidin at HIV diagnosis and mortality.
3. Whether hepcidin is a symptom of advanced disease stage and its associated inflammation, or a factor that contributes to that advanced disease stage and mortality is not clear. There is, however, evidence linking elevated hepcidin to HIV propagation,

suggesting that the association between hepcidin and mortality may be driven (in part) by a biological effect of hepcidin.

4. Hepcidin is linked with complex iron distribution that is associated with inflammation, anemia and mortality in HIV infection. This is particularly relevant since anemia is associated with poor prognoses in HIV and many people suffer from anemia prior to and during ART.

Chapter 4

1. Lower transferrin concentration was associated with a greater likelihood of TB at any time point and earlier TB progression was linked to elevated concentrations of hepcidin and ferritin in household contacts of infectious TB cases.
2. There is a plausible biological mechanism in which ‘too much iron’, or likely too much iron sequestered in macrophages as ferritin or bound to circulating transferrin, leads to increased TB susceptibility via improved efficiency of *Mtb* iron acquisition.
3. Targeted monitoring of iron homeostasis biomarker profiles in TB contacts may help to identify those at greater risk for progression to TB, which could reduce delays in TB diagnosis and/or treatment, improve TB-related health outcomes and decrease TB transmission.

Chapter 5

1. Anemia was a common co-morbidity in TB and a significant portion of it was classified as anemia of inflammation (AI). While TB treatment resolved AI, it was not sufficient to

resolve all types of anemia. Anemia with iron responsive components (e.g. Iron deficiency anemia (IDA), or IDA+AI) made up almost half of all-cause anemia at TB diagnosis and was mostly unresolved after TB treatment.

2. Resolving iron responsive anemia requires iron interventions, which are ineffective and potentially unsafe when hepcidin concentrations are high, such as at TB diagnosis. After 2 months of TB treatment, however, hepcidin concentrations decrease, opening a window of opportunity for iron intervention.
3. Routine classification of anemia type and monitoring of iron homeostasis biomarkers during TB treatment may inform timing for safe and effective iron interventions.

Recommendations for Future Work

Chapter 2

1. More data are needed from diverse settings to confirm the association between elevated hepcidin and incident TB in HIV. Studies similar to the one described conducted in different population settings and with different age groups could clarify the roles of environmental, genetic and cultural factors and would provide further insight into hepcidin's proposed biological role in incident TB in HIV.
2. Data on hepcidin concentrations are needed from HIV-seropositive populations on ART. Access to ART is increasing rapidly, resulting in decreased immune activation, immunosuppression and viral load in people living with HIV. Since hepcidin is regulated by inflammation it will be important to understand how ART affects its association with

incident TB and its proposed biological role as a factor contributing to favorable conditions for *Mtb*.

3. Further investigations into the mechanisms governing the relationship between iron and *Mtb* are warranted. If, as proposed, elevated hepcidin favors *Mtb* growth; *in vitro* studies may be able to demonstrate links between hepcidin-mediated macrophage iron sequestration and increased *Mtb* iron acquisition and/or *Mtb* propagation. These investigations could provide the biological basis for routine monitoring of hepcidin concentrations in people infected with *Mtb* and/or those living with HIV (many of whom are likely to be infected with *Mtb*).

Chapter 3

1. As with the recommendations for future research for chapter 2, more data are needed from diverse settings and population groups to confirm the association between elevated hepcidin and mortality in HIV, as well as the correlations between iron homeostasis biomarkers, anemia, immunosuppression and inflammation. In addition, data from HIV-seropositive populations on ART are needed in order to shed light on the complex relationship between inflammation, anemia, iron homeostasis and mortality in the context of ART.
2. Further research related to the hierarchy of hepcidin regulation is warranted. We know that hepcidin expression is regulated by iron status, inflammation, erythropoiesis and hypoxia, but the hierarchy is not clear and this complicates efforts to untangle the effects caused by changes in hepcidin concentration from other factors.

3. Experimental data on the effectiveness of hepcidin interventions (e.g., anti-inflammatories, hepcidin-neutralizing antibodies) is needed. These interventions seem promising from the perspective of decreasing anemia, but they may interfere with iron homeostasis and the host-pathogen competition for iron.

Chapter 4

1. Further studies that are similar to the one described in chapter 4 are needed. Future studies must have larger sample sizes, HIV-free populations, age variation and longitudinal collection of biological samples leading up to the diagnosis of TB. While the nature of TB pathogenesis makes it difficult to conduct these studies, the conclusions drawn in chapters 2 and 4 make a case for prioritizing this research.
2. Similar to the future research recommended for Chapter 2, investigation into a plausible biological mechanism in which a specific iron homeostasis profile favors *Mtb* iron acquisition is warranted. *In vitro* studies that examine siderophore iron acquisition relative to changes in host iron homeostasis may be able to elucidate a mechanism that explains the results reported in this chapter.
3. Working under the assumption that iron homeostasis biomarkers provide clinically useful information related to the likelihood of developing of active TB disease, improved technology to monitor the biomarkers should be a priority. Currently, access to laboratories capable of monitoring hepcidin, ferritin, transferrin and sTfR in low resource settings is extremely limited. Initiatives to develop cost-effective point-of-care assays for these biomarkers are needed.

Chapter 5

1. Greater efforts to match anemia interventions to causes are needed, particularly in low resource settings.
2. Anemia of inflammation is not limited to TB. It is likely that the evidence described in chapter 5 and its implications for anemia interventions are relevant for other diseases that cause inflammation. Studies investigating anemia types and iron homeostasis biomarkers in other disease contexts are warranted.
3. There is debate surrounding the safety and efficacy of iron supplementation in areas where infectious disease is endemic. Recent evidence suggests that hepcidin concentrations, which regulate dietary iron absorption as well as macrophage and enterocyte iron efflux, are the key to determining when oral iron supplementation will be safe and effective. Experimental studies that validate hepcidin's key role in this determination and identify parameters that constitute safe and effective iron supplementation are needed.
4. Studies that evaluate iron homeostasis biomarkers throughout TB treatment with increased frequency are crucial to the accurate identification of anemia intervention opportunities. Pinpointing these opportunities should lead to more targeted, more effective anemia interventions.

APPENDIX 1: ROLES OF IRON HOMEOSTASIS BIOMARKERS

| | Established iron-related function | Biological mechanisms of effect |
|-------------------------------------|--|---|
| transferrin (Tf) | The Tf protein transports absorbed ferric iron from intestinal enterocytes to cell-associated TfR. This allows iron transfer from the extracellular to intracellular environments. | Known source of <i>Mtb.</i> -acquired host iron. |
| Hemoglobin (Hb) | Hb contains the primary concentration of heme iron, typically accounting for about 65% of total body iron. | Known source of <i>M.tb.</i> -acquired host iron via <i>Mtb.</i> heme acquisition system. |
| Hepcidin | Acts as a negative regulator of iron export - stimulated via inflammation and/or sufficient iron status. | Promotes macrophage iron retention. Negative regulator of dietary iron absorption. |
| ferritin | Plasma ferritin concentrations are directly correlated with macrophage ferritin acting as an iron storage depot. | A known source of <i>Mtb.</i> -acquired iron. Ferritin concentrations are sensitive to inflammation and labile iron pool. |
| soluble transferrin receptor (sTfR) | Soluble transferrin receptor concentrations are directly associated with cellular TfR concentrations - TfR allows Tf-bound iron to enter cells. | sTfR concentrations are correlated with the amount of iron in the cellular labile iron pool (via IRP/IRE activity). |

2: ABSTRACT ACCEPTED FOR POSTER PRESENTATION AT KEYSTONE SYMPOSIA: HOST RESPONSE IN TUBERCULOSIS. WHISTLER, BRITISH COLUMBIA 2013.

Accumulation of transferrin receptor 1 (*TFRC*) mRNA in human caseous tuberculosis granuloma may affect *Mycobacterium tuberculosis*-induced lipid dysregulation

Minchella PA, Russell DG, McDermid JM

The caseous human tuberculosis (TB) granuloma is induced by a local adaptive immune response to *Mycobacterium tuberculosis* (*Mtb*) infection and subsequent necrosis of infected macrophages. It is a hallmark of active human TB disease. Studies suggest that host iron homeostasis may affect *Mtb* survival. Similarly, studies have noted that dysregulation of lipid metabolism may contribute to *Mtb* persistence. We investigated differences in genome-wide expression observed in tissue from caseous TB granulomas (3 subjects) and normal lung parenchyma using a publically accessible dataset. Using the Ensembl genome browser, we identified 100 genes involved in human iron homeostasis. Of the 100 genes identified, 48 showed significantly different levels of expression ($p < 0.05$) and 47/48 genes showed increased expression in the caseous TB granuloma. The greatest change in expression, representing a 565-fold increase, was observed for transferrin receptor 1 (*TFRC*). Based on the known regulatory mechanisms of transferrin receptor 1, this suggests that cells within the granuloma may be characterized by decreased intracellular iron – potentially affecting *Mtb*-induced lipid dysregulation. Lipid dysregulation and associated foamy macrophage formation are mediated through cholesterol efflux transporters *ABCA1* and *ABCG1*, both of which have been shown to be sensitive to intracellular iron levels. Alternatively, the increase in granuloma *TFRC* expression could be indicative of increased host iron efflux into the granuloma. As expected, ferritin (*FTL* and *FTH1*) and *SLC11A1* were up regulated, both of which have been linked to TB susceptibility. How the host and pathogen utilize essential nutrients during TB pathogenesis is important for further understanding of the transition from latency to disease.

Elevated hepcidin concentrations at HIV diagnosis are associated with increased mortality

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BACKGROUND: Studies across diverse populations have demonstrated that iron redistribution and anemia are associated with increased morbidity and mortality during HIV infection. Systemic inflammation upregulates hepcidin—blocking iron absorption, release from macrophages and availability to erythroid cells.

METHODS: Plasma hepcidin concentrations at HIV diagnosis were retrospectively measured in a subset of HIV Clinical Cohort participants based at the Medical Research Council Unit, The Gambia. Archived data and plasma samples were obtained from 196 HIV-1 and HIV-2 antiretroviral naïve participants. Hepcidin concentrations were measured using a competitive EIA (Bachem). Cox regression was used to investigate the relationship with mortality.

RESULTS: At HIV diagnosis, median age was 34 years, 55% were female, 61% were HIV-1, median absolute CD4 count was 250 cells/mm³ and participants were followed a median of 644 days. Median hepcidin concentration (interquartile) was 22.1 (3.3; 85.9) ng/ml among all participants. Hepcidin was positively correlated with plasma ferritin and transferrin saturation but inversely correlated with hemoglobin, transferrin and soluble transferrin receptor concentrations. In univariate analysis, elevated hepcidin was significantly associated with increased probability of all-cause mortality in a dose-dependent manner (upper tertile vs. lowest tertile incidence rate ratio, IRR 3.45; 95% CI = 2.19 to 5.42 and middle vs. lowest 2.14; 1.35 to 3.40). This association was only slightly attenuated by the inflammation biomarker, α -1-antichymotrypsin, and remained statistically significant. Inclusion of factors considered *a priori* to be predictors of mortality attenuated the observed association (model adjusted for absolute CD4, gender, body mass index, age at diagnosis 1.56; 0.87 to 2.80 – upper vs. lowest tertile and 1.42; 0.87 to 2.32 – middle vs. lowest tertile).

CONCLUSIONS: Elevated hepcidin concentrations were associated with increased mortality in this HIV seroprevalent cohort. Hepcidin, like other biomarkers of iron homeostasis, is associated with a complex iron redistribution phenomenon that occurs in people who are at greater risk of mortality in HIV infection. Hepcidin-mediated blockage of iron supply for erythropoiesis may contribute to the pathogenesis of HIV-associated anemia that is associated with increased morbidity and mortality. Hepcidin antagonists may be a potential target for treatment of HIV-associated iron redistribution and anemia.

Elevated concentrations of iron biomarkers are resolved by standard tuberculosis (TB) treatment

Minchella PA, Donkor S, Sutherland JS, McDermid JM

Studies suggest that iron status affects the risk of developing TB as well as TB treatment outcomes, but evidence characterizing iron status during TB treatment is limited. The objective of this research was to examine biomarkers of host iron status prior to and during TB treatment. Baseline levels of ferritin, hepcidin, soluble transferrin receptor (sTfR) were measured in 45 TB patients as well as 42 tuberculin skin test (TST)+ and 44 TST- controls in The Gambia. In TB patients, measurements of serum and plasma iron biomarkers were repeated at 2 months after initiation of standard TB treatment and again at the completion of treatment (6 months). Prior to the start of treatment, median concentrations of ferritin (164.5 ng/ml; IQR 95.65 to 251.8), hepcidin (83.98 ng/ml; IQR 43.84 to 144.9), and sTfR (29.36 nM; IQR 23.74 to 36.81) were significantly different in TB patients compared with TST+ and TST- controls. In TB patients, ferritin, hepcidin and sTfR decreased significantly during the first two months of treatment and maintained significantly lower concentrations through treatment completion. Elevated levels of iron biomarkers in TB patients appear to normalize following 2 months of TB treatment. Understanding fluctuations in iron status during TB treatment may assist with the clinical management of TB, particularly in regions with high burdens of iron malnutrition and corresponding iron supplementation programs.

APPENDIX 5: STANDARD OPERATING PROCEDURE (SOP) FOR ANALYSIS OF HEPCIDIN CONCENTRATION IN HIV+ PLASMA SAMPLES

This SOP was developed in collaboration with Dr. Andrew Armitage and Bakary Darboe for the Bachem Hepcidin-25 Enzyme immunoassay. Since this assay underwent extensive optimization, this SOP is quite detailed and includes recommendations for kit pooling.

1. Background

The **Bachem Hepcidin-25 EIA** is a competitive enzyme immunoassay for the quantitative *in vitro diagnostic* measurement of Hepcidin in plasma and serum. Hepcidin is a 25-amino acid, cysteine-rich peptide hormone, produced by the hepatocyte and has important role for iron homeostasis. Hepcidin controls plasma iron levels by regulating the absorption of iron from the intestine and the release of iron in the macrophage and hepatocyte. It is secreted in response to iron overload and inflammation. The production decreases in iron depletion. Increased hepcidin concentrations lead to decreased iron absorption and decreased hepcidin concentrations will cause increased iron release from the enterocyte and macrophages.

2. SAFETY PRECAUTIONS

- All reagents of this test kit, which contain human serum or plasma, have been tested and confirmed negative for HIV I/II, HBsAg and HCV by FDA approved procedures. All reagents, however, should be treated as potential biohazards in use and for disposal.
- Avoid contact with *Stop Solution* containing 2N HCl. It may cause skin irritation and burns.
- TMB substrate has an irritant effect on skin and mucosa. In case of possible contact, wash eyes with an abundant volume of water and skin with soap and abundant water. Wash contaminated objects before reusing them. If inhaled, take the person to open air.
- All blood samples should be treated as potentially infectious and all work done inside a certified Cat 3 Biological safety lab.
- Appropriate safety precautions should be utilized for biohazard material and the formaldehyde solution use.
- Personal protection equipments (PPE) including proper usage of Lab coats, gloves, face shield, and biological safety cabinet should be used.
- All used test tubes, plastic wares and pipette tips are also discarded in disinfectant containing Virkon™

3. Equipment and Reagents

Materials required but not provided

- A microtiter plate calibrated reader (450 ± 10 nm)
- 96-well plate shaker and washer (optional)
- Test tubes, pipettes and various other standard laboratory items

- Calibrated variable precision micropipettes and multichannel pipettes.
- Absorbent paper.
- Distilled or deionized water
- Timer
- graph paper or software for data reduction

Reagents provided

- EIA buffer concentrate (50ml 20X concentrate)
- 96-well immunoplate with acetate plate sealer
- Antiserum (lyophilized powder)
- Standard (1 ug lyophilized powder)
- Biotinylated tracer (lyophilized powder)
- Streptavidin-HRP (100ul 200X concentrate)
- TMB substrate solution (11ml TBM and H₂O₂)
- Stop solution 2N HCl (15ml)
- Standard diluents 8ml (peptide – free human serum)

Storage

- When multiple plates are required for the study, aliquoted stocks of reagents should be prepared in advance and stored at -80°C until use.
- EIA plates, 20x EIA buffer, Strep-HRP, TMB, and 2N HCl (all supplied) should be stored at 2-8°C.
- All reagents should be brought to room temperature before use.

4. Reagent Preparation:

Preparation of Stock reagents

- **We suggest that a significant portion of time is allocated for preparation of reagent stocks, and that this should probably not be the same day that the first plate is run.**
- Before starting, it is recommended that the volumes suggested below are tested for your lab's particular multichannel pipette arrangements (multichannels, reservoirs etc) – e.g. for standards, can 3 x 50µl standard be accurately pipetted from 180µl – is this excess sufficient?

Preparation of diluent and standards

- Bring components to room temperature before commencing.
- Pool standard diluent from X kits (8ml provided per kit) – mix well. [adjust quantities according to the number of samples / kits needed in the project / part of the project.]
- Reconstitute lyophilised standard (hepcidin peptide) from 1 kit in 1ml pooled diluent = 1µg/ml (some lyophilised material is likely to stick to the inside of the rubber cap; suggest

gently lifting up rubber cap, but not removing it completely - and adding 1ml diluent through the available gap).

- Invert and vortex to ensure hepcidin standard is completely dissolved.
- Spin down drops of liquid from lid by placing glass vial in 50ml falcon – to ~1500rpm.
- Carefully remove vial from falcon tube with tweezers.
- Dilute stock hepcidin standard to 25ng/ml – e.g. for 11 kits add 140µl to 5.46ml standard diluent in a 15ml falcon tube = total 5.6ml. (Note this gives an excess). Adjust volumes as necessary according to the number of assays required.
- 2-fold x 10 serial dilution – e.g. for example above, 2.8ml + 2.8ml diluent to generate 5.6ml each (for 11 kits with excess, adjust as necessary) of 25ng/ml (S1), 12.5 (S2), 6.25 (S3), 3.125 (S4), 1.5625 (S5), 0.78125 (S6), 0.391 (S7), 0.195 (S8), 0.098 (S9), 0.049 (S10).
- Pipette up and down and vortex between each dilution to mix.
- Aliquot each standard – labelled S1 – S10: 180µl per aliquot is recommended (3 x 50µl = 150µl needed for triplicate wells in assay); note that this is a 20% excess
- Also aliquot 360µl standard diluents to tubes labelled S0/Blk to add to (a) No antibody control well (Blk) and (b) no hepcidin peptide control (S0) wells on plate (i.e. 6 wells require this, not 3). [See suggested plate setup]
- Place sets of standards S1-S10 + S0/Blk in small bags -80°C freezer – 1 bag of standards per plate.
- Thaw one bag of aliquots for each plate within an hour of adding to the plate.
- It is possible to make more sets of aliquots are made than the number of kits (owing to the excess built in) – this may be worthwhile to allow for error etc (there is an excess of reagent provided – this point is true for biotinylated-hepcidin tracer and antiserum as well). Alternatively, we may be able to ‘create’ extra kits in this way (all that would be required would be extra coated ELISA plates, TMB, Strep-HRP and 2N HCl).

Preparation of serum diluent aliquots

- Remainder of standard diluent (peptide free human serum): aliquoted into 1ml and 500µl aliquots.
- Store at -80°C
- Appropriate amounts to be thawed for preparation of samples (depending on dilutions required) before each plate is run – see general points below.

Preparation of Bt-tracer and antiserum

- Bring all components to room temperature before commencing.
- Dilute EIA buffer concentrate 20x: e.g. for 11 kits, 10ml EIA buffer concentrate + 190ml sterile milliQ water (e.g. in sterile T75 tissue culture flask – normally use another bottle for routine washes; this EIA buffer is for reconstitution of Bt-tracer and antiserum). [This only

requires EIA buffer concentrate from one kit, adjust volumes if larger numbers of samples are required.]

- Reconstitute Bt-tracer (biotinylated hepcidin) and antiserum samples (rabbit anti-hepcidin antiserum) from e.g. 11 kits in 5ml EIA buffer each – total 55ml.
- Gently lift rubber cap and add 5ml buffer using 5ml pipette through gap without fully removing lid (some lyophilised peptide inevitably remains on the cap, so care required).
- Invert vial and vortex; spin in 50ml falcon tube to remove drops from rubber cap and remove from falcon tube using tweezers.
- Pool antiserum and Bt-tracer samples in e.g. 50ml falcon, or appropriate sized polypropylene vessels (polypropylene to minimise risk of peptide/protein sticking to vessel walls). Mix well. **Extreme care is required at this step as these components are not provided in significant excess – an error at this point can cost an entire set of kits – very expensive!**
- Make 3 x 1.1ml aliquots of both Bt-tracer and antiserum for each plate that is required.
- Freeze Bt-tracer and antiserum at -80°C.
- Additionally – prepare 1ml aliquots of EIA buffer prepared – to be used in the no antibody blank control wells in place of hepcidin antiserum. Store with antiserum in -80°C freezer.
- Thaw 3 aliquots per plate within an hour of use = 3.3ml (2.325ml antiserum required per plate; 75µl EIA buffer required per plate; 2.4ml Bt-tracer required for 96-well plate).

General points:

- Bring reagents to room temperature before commencing procedure (typically thaw within an hour of use).
- We have typically found that a starting 1 in 5 (e.g. 40µl + 160µl pooled diluent to give sufficient excess: 3 x 50µl diluted sample needs to be added to the plate) or 1 in 6 dilution of sample is most likely to give a reading in the linear region of the curve. Extremes may be predicted by serum ferritin data if available, as there is typically a reasonable positive correlation between ferritin and hepcidin (i.e. it might be worth diluting to a greater extent a sample known to have a very high ferritin, and vice versa for low ferritin).
- Standards / Samples can be added to / diluted in flat-bottom 96-well plates in columns 1, 4, 7 and 10 in appropriate positions (see setup below) – for transfer to the EIA plate using multichannel pipettes (50µl added).
- Use plate shakers during incubations – 60rpm as recommended.
- Ensure no precipitates are present in TMB.
- We tend to develop the colour in the dark (e.g. under a box).
- We have found that 10 or 15 minutes is sufficient for development of blue after adding TMB (point 12 recommends 30 mins to 1 hr) – in our experience it will overdevelop with longer than this. We typically stop the reaction using 2N HCl at 10 min, to develop the yellow colour which is read at 450nm within 10 minutes (it's normally ok for a little longer than this, but aim to read within 10 minutes).

- **IMPORTANT: bubbles in wells have a significant effect on the readings generated and will lead to significant inaccuracy. Ensure bubbles / foaming is not generated; if bubbles are present, use e.g. 10µl pipette tips to try to pop them.**
- Analyse using 4-parameter logistic curve fitting software (often available on software associated with ELISA plate reader).

5. Procedure

Protocol (essentially the same as Protocol V in the Bachem kit handbook)

- Bring all kit components to room temperature before commencing.
- Thaw 3 vials of 1.1ml anti-hepcidin antiserum - 25ul per well of antiserum except blank wells A1-A3 (add EIA buffer) – multichannel pipette.
- Incubate at room temperature for 1 hr on shaker.
- Thaw plasma samples for testing.
- Dilute plasma samples appropriately in diluent (from aliquots of pooled peptide-free human serum). Suggest 1 in 5 dilution as first test (e.g. 40µl + 160µl diluent – 150µl required for triplicate – 3 x 50µl) – see general point about use of flat-bottomed 96-well plate to make this more straightforward.
- Add 50ul standards S1 – S10 in appropriate wells (S9 may be omitted to allow more samples to be run) / 50ul diluted plasma samples / 50ul diluent to blank wells (A1-A3 / D4-D6).
- Incubate 2hr at room temperature on shaker.
- Thaw 3 x 1.1ml aliquots of Bt-tracer – add 25ul per well.
- Incubate overnight – 18hr – on shaker in cold room.
- Day 2: Equilibrate plate to room temperature e.g. 1 hour
- Briefly spin Streptavidin-HRP vial top get drops off lid – add 60ul to 12ml EIA buffer in e.g. 15ml falcon tube.- mix well.
- Taking care not to cross-contaminate during first wash – discard well contents – wash 5 x with 300µl EIA buffer (e.g. 2 x 150µl depending on multichannel available) – approx 30ml per wash cycle.
- Add 100ul/well Streptavidin-HRP.
- Incubate 1 hr at room temperature on shaker
- Wash 5 x with EIA buffer as above.
- Add 100µl/well TMB solution to all wells including blanks.
- Incubate 10-15 mins at room temperature in dark.
- Stop reactions with 100µl/well 2N HCl
- Read OD450 within 10 minutes – **ensure there are no bubbles present before reading.**

APPENDIX 6: STANDARD OPERATING PROCEDURE (SOP) FOR ANALYSIS OF FERRITIN CONCENTRATION

This SOP was created for the Enzyme immunoassay for *in-vitro-diagnostic* quantitative determination of ferritin in human serum kit, produced by IBL International.

Prior to start:

1. Remove samples/calibrators/controls/solutions (including HRP, wash TMB etc) from freezer/fridge at least 1-hour prior to use.
2. Prepare working solutions of the anti-ferritin-HRP conjugate and wash buffer.
Preparation of HRP: Dilute 1:50 in assay buffer (ready to use) before use (dilute 500 µl HRP in 24.5 mL of assay buffer)
Preparation of wash buffer: Dilute 1:10 in distilled or deionized water before use (50 mL of the wash buffer concentrate in 450 mL of water).
3. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.

Procedure:

4. Pipette 20 µL of each calibrator (A-F), control (1,2), QA and specimen sample into correspondingly labeled wells in duplicate.
5. Pipette 200 µL of the conjugate working solution (HRP) into each well (use a multichannel pipette and a reservoir).
6. Seal plate, incubate on a plate shaker (200 rpm) for 30 minutes at room temperature.
7. Wash the wells 5 times with 300 µL of diluted wash buffer (use multichannel pipette) per well and tap the plate firmly against absorbent paper to ensure that it is dry.
8. Pipette 150 µL of TMB substrate into each well at timed intervals.
9. Incubate on a plate shaker in the dark (cover with aluminum foil) for 13 minutes at room temperature (or until calibrator F attains dark blue color for desired OD). =
10. Pipette 50 µL of stopping solution (pipette slowly, watch for bubbles) into each well at the same timed intervals as in step 7.
11. Read the plate on a microwell plate reader at 450 nm within 20 minutes after addition of the stopping solution.
12. Plot 4PL curve and impute concentrations

APPENDIX 7: STANDARD OPERATING PROCEDURE (SOP) FOR ANALYSIS OF TRANSFERRIN (Tf) CONCENTRATION

This SOP was created for the Transferrin ELISA, produced by Cygnus Technologies.

Prior to start:

1. Bring everything to RT
2. Dilute wash concentrate to 1 liter and store at 4° C (25 ml wash concentrate + 475 distilled water)
3. Dilute samples using 3 consecutive 1:100 dilutions (for a final 1:1,000,000 dilution)
 - a. Dilution 1: 5 µl sample + 495 µl diluent
 - b. Dilution 2: 5 µl dilution 1 + 495 µl diluent
 - c. Dilution 3: 5 µl dilution 2 + 495 diluent

Procedure:

1. Pipette 50 µl standard, QA, sample into wells
2. Pipette 100 µl HRP into each well
3. Cover and incubate while shaking at 400 rpm for 2 hours (vigorously bang out liquid over absorbent paper, do not allow wash solution to remain in wells for longer than a few seconds, do not allow wells to dry before addition of TMB).
4. Wash (x4) with (300-350 µl) wash concentrate
5. Pipette 100 µl TMB into each well
6. Cover and incubate for 30 minutes (do not shake)
7. Pipette 100 µl stop solution

Read at 450/650 nm blanking on zero standard (450 for test wavelength 650 for reference wavelength)

APPENDIX 8: STANDARD OPERATING PROCEDURE (SOP) FOR ANALYSIS OF SOLUBLE TRANSFERRIN RECEPTOR (sTfR) CONCENTRATION

This SOP was created for the Quantikine IVD human sTfR immunoassay, produced by R&D Systems.

Prior to Start:

1. Bring all reagents to room temperature before use.
 2. Wash Buffer Concentrate - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.
 3. sTfR Controls - Reconstitute each vial with 200 µL of deionized or distilled water. Vortex. **Allow Controls to sit for a minimum of 30 minutes before use.** Vortex again immediately before use.
- All other reagents are ready for use.

Procedure:

1. Prepare all reagents as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 µL of sTfR Assay Diluent to each well.
4. Add 20 µL of standard, sample, QA or control per well. Ensure sample addition is uninterrupted and completed within 15 minutes. **Gently tap the plate frame to mix the well contents.** Cover with the adhesive strip provided. Incubate for 1 hour at room temperature (18 - 25° C).
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or by decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of sTfR Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate to each well. Incubate for 30 minutes at room temperature in the dark.
9. Add 100 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

“0 standard” is the plate blank; Plot with a 4PL curve*